Induced mutagenesis through gamma irradiation of embryogenic callus and selection for drought tolerance in sugarcane

by

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Declaration

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Abstract

Sugarcane is an economically important crop plant in South Africa as it serves as the primary source of sugar, fulfilling a major role in job creation, earn revenue via export, and as a value-added lignocellulosic biomass. With the current changes in climate, this crop' sustainability is threatened by limited water resources. Traditional plant breeding methods in sugarcane are hampered due to its high ploidy count and limitations linked to inflorescence and pollen production. Thus, additional methods have to be used to create new cultivars bearing drought-resistant traits. Mutation breeding through chemical and physical mutagenesis approaches were explored in this study to induce useful genomic changes linked to enhance drought tolerant traits.

Gamma radiation was used as a method of physical mutagenesis in which *Saccharum* spp. hybrid cultivars NCo310 and N58 were irradiated, with dosages between 10-40 Gy, and selected for osmotic tolerance under *in vitro* conditions. Callus cells from these two cultivars were irradiated, selected and regenerated *in vitro* on growth media containing the osmoticum, PEG6000, creating a simulated drought environment in the hope to obtain plantlets which display an enhance drought tolerance phenotype. One plantlet survived the selection regime but proved unable to survive under normal growth parameters in the glasshouse.

Radiation-induced damage was assessed by detecting and monitoring the formation of micronuclei in the irradiated sugarcane cells. Micronuclei have been considered a genotoxic marker for radiation damage in other species, but have never been shown in sugarcane. In most cases, micronuclei formation is known to be directly proportional to the amount of radiation exposure. In this study, microcnuclei were successfully detected through fluorescent microscopy using DAPI as a stain. However, in this study a linear correlation between the radiation dose and amounts of micronuclei formed could not be established. The formation of these micronuclei varied across radiation dosage and detection time after exposure, in comparison no micronuclei were detected in non-irrradiated control sugarcane tissue.

EMS (ethyl methanesulphonate) was used as a chemical mutagen in a sugarcane mutation breeding study conducted by Masoabi *et al*. (2017), which generated a number of *in vitro* osmotic tolerant plants. In the current study, three of these plantlets were multiplied *in vitro* and glasshouse pot trials setup. The aim of the experiment was to determine whether the *in vitro* osmotic tolerant plants also display an enhanced *ex vitro* drought tolerant phenotype and normal growth attributes. Plants of the M3 mutant line were significantly taller and had more sucrose and glucose in mature stalk tissue when

compared to the wildtype control plants. Under drought conditions, all mutant lines survived longer than the N19 WT (wildtype) plants. Physiological and biochemical analysis of the droughted mutant plants further detected higher relative water content in leaves, stomatal conductance and chlorophyll fluorescence suggested that the photosynthetic machinery stayed active longer, less reactive oxygen species formed and higher levels of catalase and superoxide dismutase were present in the mutant lines, when compared to the WT plants. Also, malondialdehyde levels was lower in mutant lines, which contribute to less lipid peroxide damage. These experiments showed promising results in terms of using a chemical such as EMS to induce drought tolerance in sugarcane.

Opsomming

Suikerriet is ‘n ekonomies belangrike gewasplant in Suid-Afrika aangesien dit dien as die primêre bron van suiker, dit speel ‘n belangrike rol in werkskepping, inkomste verdien via uitvoere, en as ‘n toegevoedge waarde in lignosellulose biomassa. Met die huidige verandering in klimaat word hierdie gewas se volhoubaarheid bedreig deur beperkte waterhulpbronne. Tradisionele planteteling metodes in suikerriet word bemoeilik as gevolg van hierdie gewas se hoë ploïdietelling en beperkings in terme van bloeiwyse en stuifmeel produksie. Dus moet bykomende metodes gebruik word om nuwe kultivars te kweek wat droogte verdraagsame eienskappe toon. Mutasieteling deur middel van chemiese en fisiese mutagenese word in hierdie studie ondersoek om nuttige genomiese veranderinge aan te bring wat gepaard gaan met droogteverdraagsame eienskappe.

Gammastraling is gebruik as ‘n metode van fisiese mutagenese waarin die *Saccharum* spesie hibried kultivars NCo310 en N58 bestraal is, met dosisse tussen 10-40 Gy, waarna seleksie toegepas is onder *in vitro* toestande vir osmotiese toleransie. Kallus selle van die twee kultivars is bestraal, geselekteer en gegenereer *in vitro* op groeimedia wat die osmotikum PEG6000 bevat. Die osmotikum simuleer ‘n droogtestres omgewing en die eksperiment is uitgevoer in die hoop om plantjies te verkry wat ‘n droogteverdraagsame fenotipe toon. Slegs een plantjie het die seleksie proses oorleef maar kon nie onder normale groeiparameters in die glashuis oorleef nie.

Stralingsgeïnduseerde skade is beoordeel deur die opsporing en monitering van die vorming van mikrokerne in bestraalde suikerriet selle. Mikrokerne word beskou as ‘n genotoksiese merker vir bestralingsskade in ander spesies maar is nog nooit in suikerriet getoon nie. In die meeste gevalle is mikrokernvorming bekend dat dit direk eweredig is aan die hoeveelheid bestraling waaraan die monsters blootgestel is. In hierdie studie is mikrokerne suksesvol geidentifiseer deur fluoreseerende mikroskopie deur die gebruik van DAPI as ‘n kleurmiddel. Daar kon egter nie ‘n lineêre korrelasie tussen die bestralingsdosis en die hoeveelheid mikrokerne wat vorm getrek word nie. Die vorming van mikrokerne het verskil na gelang van die bestralingsdosis en opsporingstyd na blootstelling. In vergelyking is geen mikrokerne bespeur in nie-bestraalde kontrole suikerrietmonsters.

EMS (etiel-metaansulfonaat) was as ‘n chemiese mutageen in ‘n suikerriet-mutasieteling studie wat deur Masoabi *et al*. (2017) uitgevoer is, gebruik. Hierdie studie het ‘n aantal *in vitro* plantjies wat osmotiese verdraagsaamheid toon gegenereer. In die huidige studie is drie van hierdie plante *in vitro* vermenigvuldig en gebruik om glashuispotproewe op te stel. Die doel van die eksperiment was om te bepaal of die *in vitro*-osmotiese tolerante plante ook ‘n verbeterde *ex vitro* droogtetolerante

fenotiepe en normale groei-eienskappe toon. Plante van die M3-mutantelyn was aansienlik groter en het meer sukrose en glukose gehad in volwasse stamweefsel in vergelyking met die wildetipe kontrole plante. Onder droogtestres toestande het alle mutante lyne langer oorleef as die N19 wildetipe plante. Fisiologiese en biochemiese analise van die droogte-gestresde mutante plante het verder getoon dat hoër relatiewe waterinhoud in blare, stomatale geleiding en chlorofil fluoresensie moontlik aandui dat die fotosintetiese masjinerie langer aktief gebly het as in die kontrole plante. Minder reaktiewe suurstofspesies gevorm en hoër vlakke katalase en superoksied dismutase was teenwoordig in die mutante lyne, in vergelyking met die WT plante. Laastens, malondialdehiedvlakke (MDA) was ook laer in gemuteerde lyne wat bydra tot minder lipiedperoksied skade. Hierdie eksperimente het belowende resultate getoon in terme van die gebruik van ‘n chemikalieë soos EMS om droogtetoleransie in suikerriet te induseer.

List of Abbreviations

%	Percentage
°C	Degrees Celsius
^{14}C	Carbon-14
2,4D	2,4-Dichlorophenoxyacetic acid
^{235}U	Uranium-235
^{32}P	Phosphorus-32
AFLP	Amplified fragment length polymorphism
APX	Ascorbate peroxidase
ATP	Adenosine triphosphate
BM	Breeding material
CAT	Catalase
cm	centimetres
^{60}Co	Cobalt 60
^{137}Cs	Caesium 137
DAB	Diaminobenzidine
DNA	Deoxyribonucleic acid
DT	Drought tolerance
EDTA	Ethylenediaminetetraacetic acid
EMS	Ethyl methanesulfonate
ENU	N-ethyl-N-nitrosourea
ETC	Electron transport chain
eV	Electron volts
FISH	Fluorescence *in situ* hybridization
FW	Fresh weight
GMO	Genetically modified organism
Gy	Gray
H	Hydrogen
H_2O^+	Ionised water
HMDS	Hexamethyldisilazane

IM	Induced mutant
KeV	Kiloelectron volts
LET	Linear energy transfer
M1	Second generation
Mbp	Mega base pairs
MDA	Malondialdehyde
MeV	Megaelectron volts
mm	Millimetres
MNU	N-Nitroso-N-methylurea
MS	Murashige and Skoog 1962 medium
Na_2HPO_4	Disodium phosphate
NADH	Nicotinamide adenine dinucleotide
$NADH_2$	Nicotinamide adenine dinucleotide
NaN_3	Sodium azide
NBT	Nitroblue tetrazolium
OH^-	Hydroxyl
pA	Picoampere
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
Pro	Proline
PVP	Polyvinylpyrrolidone
WW	without water
RAPD	Random amplification of polymorphic DNA
ROS	Reactive oxygen species
SNP	Single nucleotide polymorfism
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TBA	Tricarboxylic acid
TCA	Trichloroacetic acid
TILLING	Targeting induced local lesions in genomes
TVD	Top visible dewlap leaf

v/v	Number of ml per 100ml solution
w/v	Number of grams per 100ml solution
WT	Wildtype
WUE	Water use efficiency
µm	Micrometer
µM	Micromolar
µmole	Micromole

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PREFACE

General Introduction

Drought and agriculture

Currently the world's climate is changing, which is having an adverse effect on plant growth. Areas that usually received an adequate amount of water are suddenly faced with a drastic decrease in water availability in terms of rainfall. This phenomenon is confirmed by the National Oceanic Atmospheric Association (Global Climate Report, 2018), which indicates that temperatures around the globe have been steadily rising by 0.94°C since 1880, which in turn is causing changes in precipitation frequency and levels. According to MacKellar *et al* (2014) there is a clear decrease in the amount of rainfall that the country and surrounding areas have received and will continue to do so in the years to come, a statement that is also confirmed in other literature (Kruger and Nxumalo, 2018; Mason *et al*., 1999; Narisma *et al*., 2007; Newell *et al*., 1975).

This is especially worrying as South Africa, already considered a water scarce country, has a diverse array of plant crop species that require high levels of water to sustain production. This apparent variation in weather patterns due to climate change, lack of reliable and predictable rainfall and the concomitant increase in temperature and resulting elevated evaporation levels, increases the stress put on crop production across the globe.

A further consequence of unpredictable rainfall is the potentially overuse of groundwater, which could mean that even less water is available for crop production. A decrease in crop production due to water deficit will potentially result in food scarcity, cause food prices to rise, increased importing of staple food sources to counter food shortages, jeopardise numerous jobs in both the food production and processing chains, and in general affect the livelihoods of all those involved within the agricultural sector (Wilhite *et al*., 2007) Thus, as we have no power over the climate and prevailing weather patterns, scientists and plant breeders must focus on changing and/or altering the crops that we currently have, to best prepare them for adverse weather conditions.

The term drought is defined as a prolonged period where water availability is altered abnormally, for example in terms of rainfall, which causes abiotic stress and can negatively influence plant growth and development due to a change in the quantity of sufficient rain (Tate and Gustard, 2000). An extended period of dry weather will cause a shortage of water in the soil and consequently a shortage in the amount of water a plant contains. Different types of soil can retain water at different rates, depending on the soil itself and its components. It is therefore possible for certain plants to have more water

available for extended periods of time if the soil components allow it. With regards to plants themselves, drought would affect them differently depending on their ability to retain water in terms of the rate of evaporation as well as their physical and chemical attributes.

Drought and its effect on plants

Drought affects a variety of functions in plants, ranging from effects on a molecular level as well as on a morphological level. Morphological changes can include an increase in stomatal closure thus respiration rate, a decrease and eventual stopping of photosynthesis and decreases plant growth and development due to a reduced ability of cells to undergo mitosis and cell expansion. Drought can also affect the uptake of ions, reduced fruit yield and changes in root morphology (Chaves *et al*., 2003; Jaleel *et al*., 2009). On the other hand, molecular damages such as the formation of hydrogen peroxide and superoxide will be prevalent during drought (Abbas *et al*., 2014).

A decrease in available water also affects the relative water content, leaf temperature and rate of transpiration in plants and will affect their ability to adapt to water stress environments (Hassanzadeh *et al*., 2009; Shahenshah and Isoda, 2010). When leaf temperature rises, plants can't affectively control their water relations which connects to changes in stomatal conductance. Plants will try to keep stomata closed so as to limit water loss through transpiration but this will in turn affect photosynthesis and plant growth (Li *et al*., 2013). When stomata are closed the plant will not be able to take up enough carbon dioxide. If CO_2 uptake is prevented or reduced, due to closed stomata, the plant can't photosynthesize and produce energy for optimal growth. The plant therefore needs to find a balance to prevent water loss through open stomata but also continue the uptake of CO_2 for photosynthesis to occur.

When a plant can no longer balance photosynthesis and water loss, excessive amounts of reactive oxygen species (ROS) will be generated. ROS, such as superoxide anion radicals (O^{2-}) and hydrogen peroxide (H_2O_2), react with components in a cell causing tissue injury through oxidative damage (Sharma *et al*., 2012). To counter ROS, the plant produces free radical scavengers such as antioxidants (Birben *et al*., 2012). However, under severe abiotic stress conditions, the generation of ROS will overwhelm the capacity of antioxidant defence systems leading to DNA, membrane, sugars and proteins damage and eventually plant death (Schieber and Chandel, 2014). The protective scavenging system comprises of enzymatic components such as superoxide dismutase (SOD), catalase and ascorbate peroxidase (APX) and non-enzymatic components such as reduced ascorbic acid and glutathione (Waśkiewicz *et al*., 2014). The ability of a plant to maintain these defence systems and use them to their full extent will enable a plant to scavenge toxic components to protect the plant from

oxidative stress. In addition, plants can attempt to regulate the osmotic balance in the plant cells through the formation of osmolytes.

Osmotic adjustment refers to the ability of the plant's cells to decrease their osmotic potential, which in turn results in an increase in the maintenance of turgor pressure as well as the gradient for water uptake (Farooq *et al*., 2009). Osmolytes such as sugars, amino acids or small proteins can limit the loss of water by binding to water molecules and protect DNA and proteins from free radicals. In addition, osmolytes can bind to membranes to stabilise cell structure (Nahar *et al*., 2016). During water deficit conditions, the disruptions of membranes can be caused by the decrease in cellular volume, which can lead to the increase viscosity of cytoplasmic elements and the fusion of membranes as well as protein denaturation. Thus, in plants it can be seen that the machinery to protect against environmental stresses includes a number of physiological and biochemical processes.

Strategic adaptations of plants to counter drought

There are mainly three strategies a drought resistant plant employs to help deal with water stress, these include escape, avoidance and tolerance (Basu *et al*., 2016). Drought escape refers to the plant's ability to shorten its life cycle to circumvent the time in which water is not readily available. This will include rapid phenological development such as rapid growth so as to achieve the highest rate of development before drought is experienced (Shavrukov *et al*., 2017). Secondly, avoidance speaks to the mechanisms a plant has in place to maintain a relatively high tissue water content despite diminished soil water content. This is achieved through adaptive traits to minimise water loss and optimize water uptake. Included are changes such as increase in root growth as well as a reduction in transpiration and vegetative growth, all in an effort to increase water use efficiency (Kooyers, 2015). Lastly, tolerance refer to the ability of plants to endure low water content in tissue through osmotic adjustment to control cell turgor and cellular elasticity (Touchette *et al*., 2007).

Sugarcane an important economical crop species in South Africa

Sugarcane, *Saccharum* spp. hybrid, produces high levels of sucrose, which can be harvested not only for the use of sugar, but also for the generation of biofuels. Sugarcane is a monocotyledonous plant that is mostly farmed in sub-tropic and tropic locations around the world such as Brazil, India as well as in South Africa (Sant'anna *et al*., 2013). According to The South African Sugar Association (SASA) (Sasa.org.za, 2018), South Africa's sugarcane industry employs an estimated 24 000 independent sugarcane farmers, that produce about an average of 20 million tons of cane on about 370 000

hectares of land per annum. This industry makes a large contribution to the South African economy, upwards of R12 billion a year.

The influence of drought on sugarcane production

In South Africa sugarcane crop production relies not only on irrigation but on regular rainfall (Carr and Knox, 2011). It is said that sugarcane utilises a ML (megaliter) of irrigation water for an estimated maximum of 12 tons of cane (Ferreira *et al*., 2017). This is a strong indication that sugarcane relies heavily on water availability to produce sufficient yield. However, in recent years the SA sugar industry experienced below average rainfall, with an industry average of 20% below the long-term mean. Under these unfavourable conditions, 15.07 million tons of cane were produced in the 2016/2017 season, yielding 1.55 million tons of sugar; production figures slightly higher than those of the 2015/2016 season but below the five-year season mean (Sasa.org.za, 2018). Furthermore, climate change modelling studies conducted on behalf of the sugarcane industry reveal a high likelihood of mid-century scenarios in which extended periods of low rainfall alternate with extreme rainfall events (Department Agriculture, Forestry and Fisheries, 2018). Hence, predicted climatic conditions indicate the necessity of sugarcane varieties with increased drought tolerance. It is therefore important to investigate possible methods for the sustainable growth of sugarcane as a crop in a future environment prone to unpredictable precipitation level due to climate changes.

Attempts towards improving sugarcane's properties have been done in the past using conventional plant breeding, biotechnological approaches and genetic engineering. The development through conventional breeding of sugarcane varieties with improved resilience to abiotic stress through the application of conventional breeding tactics, present significant challenges. Modern sugarcane cultivars (2n = 100-130), are derived from hybridisation events between two primary progenitor *Saccharum* species, namely *S. spontaneum*, and *S. officinarum* (Nair, 1975). The result is a highly heterozygous genome that is highly polyploid and aneuploid (Mancini *et al*., 2018). Furthermore, as sugarcane is clonally propagated, highly heterozygous and suffers from inbreeding depression, sugarcane breeders struggle to utilise the traditional breeding approaches used for the development of superior varieties. Such genetic and genomic complexity also impeded the development of genomics-based breeding technologies that have been so successfully deployed for other crop species (Hoang *et al*., 2015). Consequently, seeking alternative technologies to enable the breeding of sugarcane varieties with increased tolerance towards water-deficit stress is of the upmost importance. One potential avenue for enhancing drought tolerance in sugarcane is by inducing genetic diversity through mutation breeding (Pathirana, 2011). Thus, this project will focus on sugarcane and our ability

to change its genetic material to establish sugarcane genotypes that will be able to accommodate changes in climate, specifically drought.

Aim and Objectives

The aim of this project is to create drought tolerant sugarcane plants by using random mutation induction breeding methods. To achieve this aim this study consisted out of two parts with the following objectives:

Part 1 – Random mutation induction through the use of gamma irradiation

1. *In vitro* explant initiation for commercial sugarcane cultivars NCo310, N51 and N58
2. Determine optimal gamma dosage, ranging between 10 to 40 Gy, for mutation induction in NCo310 callus and leaf disc tissue. Assess callus growth, somatic embryogenesis, root formation and nuclear damage at the different irradiation dosages.
3. Irradiation of *in vitro* plant material, at optimal dosage, of cultivars NCo310, N51 and N58 followed by *in vitro* selection for osmotic tolerance.

Part 2 – Analysis of drought tolerance in mutant sugarcane lines generated though chemical mutagenesis

1. *In vitro* multiplication of mutant lines and setup of greenhouse pot trial.
2. Initiate drought pot trial followed by physiological and biochemical analysis of stressed mutant and control sugarcane lines.

Chapter Layout

This MSc thesis contains four chapters.

Chapter 1 reviews existing literature on mutation breeding, including chemical and physical mutagenesis.

The main focus of **Chapter 2** is on the creation of gamma-radiated mutant sugarcane plants and their ability to survive drought induced environments compared to the WT.

Chapter 3 focuses on the detection of micronuclei to assess physical mutagenesis damage by using different microscopy techniques.

Chapter 4 describes the evaluation of EMS sugarcane plants in a drought trial in terms of physical and biological differences seen between WT plants and those mutated with the chemical EMS.

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Chapter 1: LITERATURE REVIEW

1.1 Mutation breeding

Mutation breeding is the process where random mutations are induced in the DNA of a crop plant, resulting in desirable traits, without sacrificing the growth and development of the plant in question (IAEA.org, 2019). Mutation breeding is based on two fundamental concepts, namely mutation induction and mutation detection (FAO/IAEA, 2019). Mutation induction involves the treatment of plant tissue, mostly seeds, with chemical or physical mutagens. This is followed by selection for desirable traits in the resulting mutants. Plants created through mutagenesis are called mutants or mutagenic plants. Mutations are induced in the hope that the damage to the DNA will result in an effective form of genetic variation (Wilde, 2015). Therefore, through mutation induction the genetic base of crop germplasm can be broadened, to be used directly as new varieties or indirectly as sources of new variation in breeding programs.

Mutation breeding started as early as the 1900's, where in 1966 the first mutagen-treated wheat genotypes, achieved by gamma radiation, were registered on the FAO/IAEA mutant variety database (FAO, 2018). Since then over 2000 cultivars have been registered that had been mutated using physical mutagenesis versus over 200 cultivars using chemical mutagenesis. The plants in question include wheat, rice, soybean, maize, lentils and centipede grass to name a few, while the mutagen treatments include physical: gamma radiation, carbon ion beams, neutrons, x-rays and chemical: ethyl methanesulfonate (EMS), sodium azide and beryllium oxide (FAO, 2018).

These mutagenic plants have shown a wide variety of positive attributes gained through the mutation induced by the various methods. These favourable traits include, enhanced tolerance to adverse environmental factors, such as drought, high salt content, low levels of essential compounds, and late flowering/earlier flowering, better stature, more tillers, earlier maturity, improved seed production, stem rust resistance and other fungal resistance as well as yellow mosaic virus disease resistance and herbicide tolerance (Oladosu et al., 2016). The positive outcomes of mutagenesis have also seen nutritional value increase in certain plants, such as increases in starch in mung bean, macronutrients in rice, soybean and maize and increases in protein content in rice and wheat (FAO, 2018).

When using mutation breeding to enhance drought tolerance, the FAO database has 87 registered mutant genotypes, which include species such as rice and peas. These mutagenic plants were generated using methods ranging from gamma radiation to chemical mutagenesis. Of these 87 genotypes, 48 drought tolerant plant genotypes, including soybean and alfalfa, were created using gamma radiation (FAO, 2018).

1.2 Physical mutagenesis

Physical mutagenesis through radiation can be divided into two sections namely, ionizing radiation and non-ionising radiation. The latter refers to a type of radiation which does not contain enough energy to ionize an atom, but instead merely deposits energy in the material it passes through (Reisz *et al.,* 2014). Ionizing radiation on the other hand is electromagnetic waves that have enough energy to remove electrons from atoms and cause them to be unstable (Suprasanna *et al*., 2015).

Ionising radiation includes X-rays, gamma rays, ion beams and neutrons (Suprasanna *et al*., 2015). Ionizing radiation, such as gamma irradiation, travels at the speed of light and is able to penetrate material to deep levels, such as through the entire human body. It can only be stopped when it is blocked by a thick layer of material, such as several centimetres of lead or several metres of concrete. This form of radiation is also known to damage DNA directly or indirectly by creating free radicals (Desouky *et al*., 2015). On the other hand, non-ionising radiation includes microwaves and lasers. The energy emitted through these radiation types tends to be of longer wavelengths, which usually have enough energy to excite molecules but not remove electrons (Sheetz, 2015). Traditionally, mutation breeding in plants mostly uses ionising radiation for mutation induction, since non-ionising radiation is considered too weak to induce sufficient molecular changes in cells.

1.2.1 Gamma radiation

Gamma radiation is electromagnetic radiation produced from sources such as ^{60}Co (Cobalt-60) or ^{137}Cs (Caesium-137) (Oladosu *et al*., 2015). As a form of ionising radiation, gamma rays have a shorter wave length and therefore are extremely harmful with photons that can penetrate through several layers of a material/tissue (Suprasanna *et al*., 2015). These photons are the most energetic on the electromagnetic spectrum and can cause extensive damage due to their energy, which is upwards of 100 keV. The penetrative abilities of gamma irradiation is especially relevant in mutation breeding in

plants. Plant cells gather on top of each other, different to human cells occurring within distinct layers, and therefore require more energy in terms of radiation to evenly distribute the photons necessary to create mutations in all cells (Coates *et al*., 1969).

Using gamma radiation for mutations induction cause large deletions in the genome and changes in chromosomes of the radiated cells (Chaudhary and Chaudhary, 2014). This damage to DNA occurs either directly or indirectly. Direct damage refers to damage to the DNA by ionisation, while indirect damage occurs due to the formation of free radicals, atoms or groups of atoms with an unpaired electron (Desouky *et al*., 2015; Kovacs and Keresztes, 2002). This form of radiation has been used to induce mutations that have made many crop plants more tolerant towards biotic and abiotic stress (Table 1.1) (Nikam *et al*., 2015; Patade *et al*., 2008). It is the most frequently used physical mutagen and accounts for 64% of the developed mutant varieties (Amiri-Tiliouine *et al*., 2018).

Table 1.1: A summary of commercially important varieties from the Poaceae family, registered on the FAO (2018) database, created through mutation breeding using gamma radiation and specifically selected for enhanced drought tolerance.

SPECIES	MUTANT VARIETY ID	DOSAGE	TISSUE TYPE RADIATED	COUNTRY OF ORIGIN	IMPROVEMENT	DEVELOPMENT TYPE
RICE (*ORYZA SATIVA)*	607	200 Gy	Seeds	China	Increased DT, larger panicle, smaller leaves	Direct use of IM
	2395	200 Gy	Seeds	Philippines	Increased DT and yield	Direct use of IM
	1078	400 Gy	Seeds	Indonesia	Increased DT and yield, tolerance to low pH and fungal disease	Direct use of IM
	1267	150 Gy		Thailand	Increased DT, early maturity	Direct use of IM

WHEAT (*TRITICUM AESTIVUM*)	4816	120-250 Gy	Grains	Ukraine	Increased DT and yield, shorter stems, higher protein content	Direct use of IM
	2647	1.5 Gy	Pollen	China	Increased DT, yield and salt tolerance	Mutagenic treatment of BM
	635	200 Gy		China	Increased DT, increased resistance to stripe rust and earlier maturity	Direct use of IM
	3375	Unknown		Kenya	Increase DT, WUE, quality and yield, wheat rust resistance	Direct use of IM
	678	Unknown		China	Increase in DT and quality	Crossing with one mutant
	679	100 Gy	Seeds	China	Increased DT, stripe resistance	Mutagenic treatment of BM
	681	200 Gy	Seeds	China	Increased DT, better tillering	Mutagenic treatment of BM
	644	200 Gy	Seeds	China	Increased DT, resistance to hot and dry winds	Mutagenic treatment of BM
	1195	1400 Gy		Pakistan	Increase in DT, disease resistance, dwarfed growth	Direct use of IM
	719	80 Gy	Seeds	China	Increased DT, increased yield and resistance to lodging	Mutagenic treatment of BM

	687	Unknown		China	Increased DT, better yield and earlier maturity	Crossing with one mutant
	659	350 Gy	Seeds	China	Increased DT and resistance to lodging and hot dry winds, as well as earlier maturity	Mutagenic treatment of BM
	896	150 Gy		China	Increase in DT, and low temperatures	Direct use of IM
	660	300 Gy		China	Increase in DT and resistance to stripe rust	Direct use of IM
MILLET (*SETARIA* SP.)	137	250 Gy		China	Increase in DT, higher quality and yield and wider adaptability	Direct use of IM
	136	100Gy		China	Increase in DT	Direct use of IM
BARLEY (*HORDEUM VULGARE*)	1085	200 Gy		Iraq	Increase in DT, higher yield and resistance to lodging and mildew	Direct use of IM
	3149	100 Gy		Syrian Arab Republic	Increase in DT, lodging resistance and higher yield	Direct use of IM
	1269	150 Gy		Turkey	Increase in DT, larger seeds and increased tolerance to low temperatures	Direct use of IM

DT = Drought tolerance; IM = Induced mutant; BM = Breeding material; WUE = Water use efficiency

1.2.2 Alpha and beta radiation

In comparison, alpha radiation, using alpha particles derived from radioisotopes, is heavy and slow and cannot penetrate through several layers of cells (small fraction of a mm), and has an energy output of 2-9 MeV. This type of radiation can be stopped by as little as a human skin layer, and has little effect in terms of radiological damage (Suprasanna *et al*., 2015). This is due to the energy from the source being absorbed very quickly, thus not allowing further penetration through cell layers (Szymczak, 2012).

Beta radiation is produced from particle accelerators or radioisotopes from sources such as ^{32}P or ^{14}C. It is not as effective as gamma radiation due to similar energy profiles to alpha radiation, resulting in limited cell damage. Beta radiation exhibits an energy level of several MeV and can penetrate to a few centimetres (Suprasanna *et al*., 2015). The effect of this form of radiation has been used to delayed seed germination and decrease plant growth (Roy, 2015).

1.2.3 X-rays

X-rays, the first physical mutagen to be used to induce mutations, also falls within the ionising radiation spectrum. X-rays are similar to gamma radiation in that they emit electromagnetic radiation. The difference between these two forms of radiation is that X-ray energy varies between 50-300 keV, while gamma radiation can be upwards of 1 MeV, this means that X-rays can only penetrate tissue from a few millimetres to a couple of centimetres (Shu, 2012). Mutations caused by X-rays include damage to DNA, but to a lesser extent than that of gamma radiation. Some changes that have been recorded in plants due to the usage of X-rays include changes in seed content and flesh colour of fruit (FAO, 2018).

1.2.4 Neutrons

Neutrons are produced in nuclear reactors from sources such as ^{235}U and can be fast, slow or thermal uncharged particles (Oladosu *et al*., 2015). Neutrons exhibit energy levels from several MeV to just below 1 eV, and can penetrate objects to several centimetres. Neutrons are believed to be 'stronger' than gamma rays as they seem to cause deletions on a kilo-base level, resulting in serious damage to chromosomes and are therefore considered a less favourable source for induced mutagenesis (Shu, 2012). Currently, three clementine cultivars, generated using neutron radiation, have been registered with the FAO, where their increased attributes consist of few to no seeds, larger fruit and easier to peel skins (FAO, 2018).

1.2.5 Linear energy transfer

Ion beams, produced by particle accelerators, are positively charged particles travelling along a path that vary in mass from a simple proton to a uranium atom (Abe *et al*., 2002). These particles travel at high speed, at around 20% to 80% of the speed of light, which then form high linear energy transfer (LET) radiation. LET is responsible for causing biological effects at high levels that can result in chromosomal aberrations and as such can have lethal results. High LET is especially known to result in double stranded breaks, which are less repairable than gamma radiation, which is a low LET, due to more energy being deposited in the tissue cells (Averbeck *et al*., 2016; Hunter and Muirhead, 2009)

X-rays and gamma radiation also exhibit LET, although at a lower level than ion beams. Their energy range is 0.2 keV/µm (low-LET radiation) in comparison to ion beams which is between 23 keV/µm and 640 keV/µm (high-LET radiation), thus causing extensive ionisation damage (Shu, 2012). These high level LET beams can affect cells in specific areas with a more compacted ionisation. Low velocity with a highly charged ion beam is preferred for high LET production in terms of mutation breeding (Kazama *et al*., 2011). When working with ion beams the ideal radiation dosage, also measured in Gy, need to be determined and reflect the highest possible rate of mutation at a certain location which is not lethal to the exposed cells. Beams which are heavy-ion based are mostly used on higher plants as these produce mutations at a high frequency with low dosages and result in a high survival rate (Shu, 2012). These ion beams have been used to create new cultivars of chrysanthemum, tomato, cherry, rice, wheat, carnation, and sweet potato, which are registered on the FAO website. These new cultivars include a variety of beneficial mutations, including: resistance to fungal and bacterial diseases, flower colour and shape, grain/seed yield and altered maturity (FAO, 2018).

1.3 Chemical mutagenesis

Chemical mutagens, when applied to cells, cause mostly point mutations instead of large chromosomal deletions or insertions (Spencer-Lopes *et al.*, 2018). However, the mutagenic effect of a chemical relies on the initially induced damage in the DNA as well as any DNA repair mechanisms of the host plant cells. Chemical mutagens include chemicals such as: ethyleneimine, dimethyl sulfate, ethyl methanesulfonate (EMS), ethylnitrosourea (ENU) and methylnitrosourea (MNU). These chemicals all form part of a class of alkylating agents, which replace a hydrogen atom with an alkyl group (Hodgson *et al*., 2015). These alkylating agents are the main chemical mutagens currently used for crop improvement and are responsible for over 80% of newly registered mutant plant varieties on the IAEA database obtained via chemical mutagenesis. The use of these chemicals in mutation

breeding is very popular due to their effectiveness, ease of handling and the detoxification process for disposal (Spencer-Lopes *et al.*, 2018).

1.3.1 Alkylating agents

The most commonly used alkylating agent is EMS and to a lesser extend MNU. EMS is a mono-functional ethylating agent that is mutagenic to a wide range of organisms, including plants (Amini, 2014). It specifically induces alkylation on either guanine or thymine bases in the DNA molecule and adds an ethyl group. This can cause the DNA machinery to misidentify the base as an adenine or cytosine, which is commonly seen with EMS treatment, when GC to AT base pairs transitions occur (Griffiths *et al*., 2000). Numerous studies have shown that EMS mutations occur at high frequency but are distributed randomly across the genome with specific bias towards guanine residues in the R**G**CG context, where R is A or G (Mohd-Yusoff *et al*., 2015). Single base pair changes in turn result in loss-of-function or gain-of-function phenotypes due to altered protein function or structure in the plants. The frequency of mutation induction by EMS can vary greatly, from as frequently as 1 in every 23 kb in wheat to as low as 1 in every 1000 kb in barley (Caldwell *et al.,* 2004; Dong *et al*., 2009). According to the FAO, EMS has been used in the past to create new cultivars of rice, wheat, common bean, lentil, barley, soybean and tomato to name a few. These cultivars showed mutations that were beneficial in terms of: fruit shape, vitamin content, early maturity, flower/seed colour and shape and higher yield.

Chemicals such as methylnitrosourea (NMU) can produce about one mutation for every 140 kb of DNA, depending on the dosage and the plant species (Griffiths *et al*., 2000; Suprasanna *et al*., 2015). NMU has been used for mutation induction in especially crop species such as rice, maize and wheat, where mutations have been induced that bring about changes in yield, maturity and resistance to lodging (FAO, 2018).

1.3.2 Azides

Azides, such as sodium azide, are a mutagen group of mutagenic chemicals which react with DNA by adding methyl or ethyl groups to the purines and pyrimidines in the structure. Sodium azide is a very effective mutagen and has been seen to induce few chromosome abnormalities with GC to AT transitions also being the predominate mutation type but with a sequence bias of G**G**R compared to EMS (Olsen *et al*., 1993; Tai *et al*., 2016). Sodium azide is an inhibitor of cellular respiratory processes in living cells, it plays a role in DNA excision repair and inhibits certain antioxidants such as catalase (Gruszka *et al.*, 2012; Velemínský and Angelis, 1987). The mutagenic effect of this chemical greatly

depends on the acidity of the treatment solution and should be applied at low pH (Gruszka *et al*., 2012). To date only one mutated species, namely barley, has been registered with the FAO that has been developed through the use of sodium azide. This genotype displayed an increased tolerance to low temperatures, resistance to stem rust and mildew as well as higher lysine and protein content (FAO, 2018).

1.4 Damage effects of physical and chemical mutagenesis

In any mutation induction breeding experiment, a mutagen must be given at a dose that results in low plant injury but induces a workable frequency of mutations for target traits. The response of plants and plant cells to physical and chemical mutagens is influenced by numerous factors. These factors influence the effectiveness and efficiency of mutagens to damage the DNA of plant cells. The most important modifying factors are the oxygen and water content of the exposed plant cells (in the case of physical mutagens), the development stage of the exposed plant tissue, plant genotype, the plant genome size and organization, and the dose rate of the mutagen (Oladosu *et al*., 2015). For example, rapidly growing tissue, where cell division and nuclear division (mitosis) are active, will be much more sensitive towards mutagenesis than dormant tissue, such as seeds (Lagoda *et al*., 2012). The primary site of mutagenesis, namely the cell nucleus, also influences mutation-sensitivity. This is due, firstly to the average nuclear volume, secondly, chromosome number, and thirdly chromosome size, which all differ between species. In general, higher chromosome numbers result in higher resistance and larger chromosomes in higher sensitivity towards mutagenesis (Datta, 2014). Mutation dosage, in general in the context of chemical mutagenesis, depends on the concentration and duration of the chemical treatment and the optimal dose depends on the plant species, target tissue and applied mutagen (Spencer-Lopes *et al*., 2018).

Mutagenesis can directly or indirectly affect plant cells and cause damage to DNA molecules (Figure 1.1). Directly, the mutagens damage the DNA by making changes in the sequence, which can either be intragenic, referring to point mutations within a gene; intergenic, such as deletions and duplication of DNA; or changes in chromosome number (Caplin and Willey, 2018). Numerous studies confirmed the presence of these types of direct DNA damage in plant genomes due to physical and chemical mutagenesis (Kwasniewska, 2019). For example, in rice exposed to radiation up to 9% of the gene sequences were altered (Cheng *et al*., 2014), while also in rice, chemical mutagenesis induced deleterious mutations in as much as 2600 genes (Henry *et al*., 2014). In general, the higher the chromosome number of the plant, the higher the resistance to the mutagens due to chromosomes or

chromosome parts that compensate for mutations (Datta, 2014). However, indirectly there are other ways that mutagens can cause damage, such as through oxidation. A study done on radiation-induced mutagenesis in petunia, by Doná *et al*. (2013), set the oxidation of nuclei acids at 10 to 15% of all DNA damage. However, a substantial amount of this damage caused by radiation mutagenesis is quickly repaired by the plant (Caplin and Willey, 2018). In contrast, damaged nuclei after chemical mutagen exposure in general takes much longer to be repaired in plant cells (Lanier *et al*., 2015). Indirect means of DNA damage through physical mutagenesis is briefly discussed in the following sections and summarised in Figure 1.1.

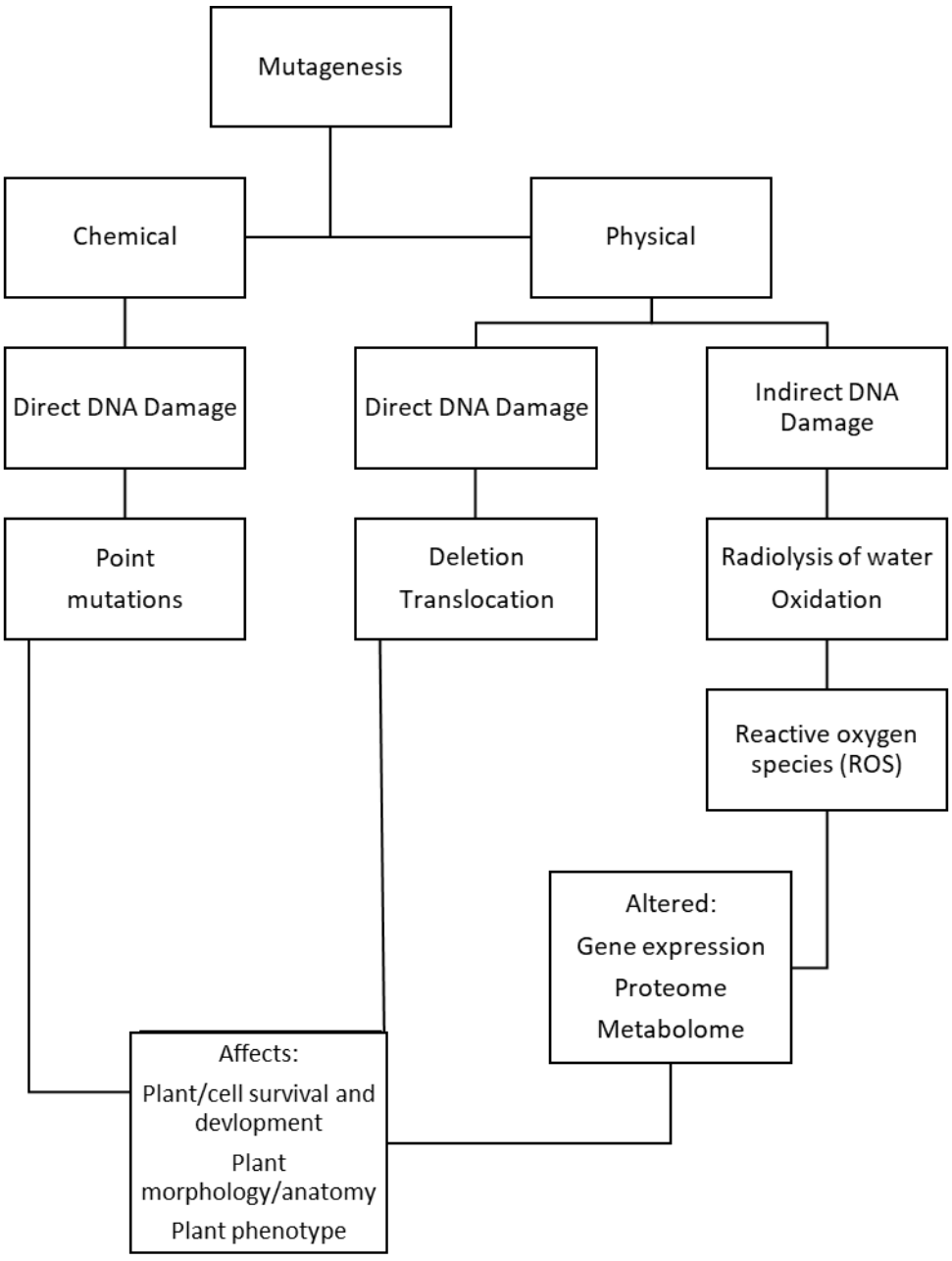

Figure 1.1: A diagram illustrating the directly and indirectly effects of mutagenesis on plant cells that can cause damage to DNA and other cellular structures resulting in changes in gene expression, proteome and metabolite profiles and over all morphological and phenotypical alterations in the plants.

1.4.1 Water content

Plant cells contain high levels of water, for example sugarcane consists of an estimated 70% water (Mauri *et al*., 2017). When plant cells are being exposed to physical mutagens, the radiation makes contact with the water and produces free radicals (Reisz *et al*., 2014). Free radicals are atoms that are unstable, due to the loss of an electron. Therefore, these molecules are extremely reactive, which leads to them either taking or giving an electron away (Lobo *et al*., 2010). Hydroxyl (OH^-) and hydrogen (H^+) radicals and ionised water (H_2O^+) are examples of free radicals that are formed due to the interaction between the radiation and the water content in cells. OH^- radicals are short living reactive molecules that can cause various forms of cell damage or even cell death (Lee *et al*., 2009). In general, these free radicals can cause damage to DNA, proteins and lipids and the higher the water content of the target tissue, the greater the related damage.

Plants tends to avoid the formation of excessive amounts of free radicals under normal circumstances through the production of compounds which can circumvent this negative occurrence, such as the production of antioxidants (Wu and Cederbaum, 2003). Even irradiated plant cells will form increased amounts of antioxidants in an attempt to limit irradiation-induced damage due to free radical formation. For example, when bunchgrass was irradiated and pine trees exposed to radiation, increased amounts of catalase, an antioxidant, were synthesized (Volkova *et al*., 2017; Zaka *et al*., 2002). However, this might not be true for all plant species or vary according to the life stage of the irradiated plant tissue or dose rates (Van de Walle *et al.,* 2016). Van de Walle *et al*. reported varying levels of antioxidants in irradiated *Arabidopsis* seedlings over two generations.

With regards to irradiation, seeds seem to be more resistant towards high irradiation dosages since they contain less water than, for example, callus. Thus, less free radicals will be formed due to the interaction between the gamma radiation and the water molecules in the desiccated seeds. When seeds are radiated with gamma rays, high dosages between 100 to 1000 Gy will be used, while markedly lower gamma irradiation dosages are used on callus, usually lower than 100 Gy, due to the high water content in the callus cells (Ambavane *et al*., 2015; FAO, 2018; Sarduie-Nasab *et al.*, 2010).

1.4.2 Oxygen content

Oxygen is also a modifier of radiation sensitivity and in general the biological effects of radiation are greater if the oxygen levels are high in the irradiated cells (Naito *et al*., 2004). The oxygen content in cells plays a role in the production of oxygen-derived free radicals, these radicals can include hydrogen peroxide and superoxide anion molecules and oxygen singlets (Lobo *et al*., 2010). These reactive oxygen species (ROS), as with water derived free radicals, are very reactive and unstable and are present in the nucleus and membranes of cells and can damage DNA, lipids, carbohydrates and proteins (Lobo *et al*., 2010). ROS induce changes in the functions and properties of proteins, such as the damage done to protein thiols, which results in an imbalance in calcium homeostasis in cells (Orrenius *et al*., 1992). In terms of lipids, they undergo peroxidation which leads to membranes being structurally different or compromised with regards to function, which can cause membrane leakage of cellular components (Jambunathan, 2010). In addition, damage to the mitochondria can also occur due to mutagenic treatments. Mitochondria can use up to 90% of the organism's oxygen, thus making them places rich in oxygen-derived free radicals and is therefore a hot spot for ROS formation. This reactive area is created by several components namely: the tricarboxylic acid (TCA) cycle, the electron transport chain (ETC) as well as oxidative phosphorylation (Wang *et al.,* 2001).

The TCA cycle takes place in the mitochondria, where the intermediates in the reaction to create NADH (nicotinamide adenine dinucleotide) and $FADH_2$ (flavin adenine dinucleotide), are electron rich and can enter the ETC to be used in the production of ATP (adenosine triphosphate) (Berg *et al*., 2002). The ETC consists of complexes that have several oxidation-reduction reactions where oxygen is reduced to water in its role as the last electron acceptor. Lastly, with the electron transfer that occurs, an H+ is moved from the mitochondrial matrix to the inter-membrane space, where this reaction causes a proton gradient that aids in the generation of ATP by using oxidative phosphorylation (Lodish *et al*., 2000). Thus, as this area is energy and oxygen rich, it houses many ROS products, such as superoxide, which is created due to a reduction of oxygen initialised by the leakage of electrons from the complexes in the ETC. Therefore, the extra damage caused by mutagens in terms of causing leakage of the mitochondrial membranes can lead to damage in mitochondrial DNA and change the coding for essential proteins for component function (Azzam *et al*., 2012).

1.4.3 Biological factors

Induced mutations, both oxidative additions and other changes to DNA, are mostly not homozygous and are originally one-cell events. Thus, M_1 plants are usually regarded as chimeric plants where

individual cells have different genotypes (Spencer-Lopes *et al*., 2018). Therefore, the selection for induced traits should be carried out in early mutant generations or desired combinations of alleles are likely to be lost in advanced generations (Amri-Tiliouine *et al*., 2018). The most prominent direct effects of mutagens are growth retardation, sterility or death of the M_1 plants (Hollaender, 1984; Meinke and Sussex, 1979). If the M_1 plants survive, aberrations at meiosis, mitosis (cell division), pollen, embryo or seed development are often seen (Spencer-Lopes *et al*., 2018). For example, in a study done on bamboo (*Bambusa arundinacea)* it was found that seeds which were irradiated showed a decrease in germination as the dosage of irradiation was increased (Lokesha *et al*., 1992). In *Arabidposis* seeds, as the irradiation dosages increased, increased changes in seed development and altered meiosis patterns were seen (Yang *et al*., 2004).

Plant morphology and development can also be altered by mutagenesis. Changes such as shoot growth (length and diameter), plant height, tuber, root, flower and bulb development were seen in potato (Hamideldin and Hussien, 2013), garlic (Mostafa *et al*., 2015), *Arabidopsis* (Nagata *et al.,* 2004), and chickpea (Amri-Tiliouine *et al*., 2018) when exposed to gamma radiation, to name but a few.

At the chemical level, the biological effects of free radicals responsible for physiological disorders can be due to DNA, protein, lipid and carbohydrate damage. For example, mutagenesis resulted in changed protein profiles, with special reference to phosphorylation patterns of the proteome, in *Arabidopsis* plants exposed to irradiation (Roitinger *et al*., 2015), and metabolite profiles changes in rice exposed to radiation (Hwang *et al*., 2015). ROS can also trigger the transcription of specific genes as a response to stress caused by the mutagen. This is often observed in the stimulated germination rate at lower irradiation doses or the increase in assimilating pigments (chlorophyll and carotenoids) (Spencer-Lopes *et al*., 2018). In addition, microarray analysis revealed extensive transcriptomic changes in rice plants when irradiated with gamma rays (Batista *et al*., 2008). As of yet, no clear picture exist as to which pathways or processes are most affected by changes in gene expression due to mutagenesis. Indications are that altered gene expression generally includes DNA repair and anti-oxidant defence pathways (Kovalchuk *et al*., 2007), but these are not the only ones involved. Studies detected changes in carbohydrate metabolism (Hwang *et al*., 2014), anti-oxidants (Van Hoeck *et al*., 2017) and photosynthetic proteins (Park *et al*., 2015), to name but a few. What is clear is that these changes in gene expression are especially linked to the mutagen dosage, applied in a chronic or acute dosage (when using irradiation), and the stage of plant development. For example, when Biermans *et al.* (2015) exposed *Arabidopsis* seedlings, at different stages of development, to chronic radiation, these plants responded in different ways with regards to growth and showed decreasing sensitivity towards radiation with increasing seedling age, which was linked to DNA repair and control at a transcriptional level.

1.4.4 Gamma irradiation effects on plant cells

The effect of gamma radiation on plant cells/tissue is well described. Gamma radiation can cause mutations in the DNA structure of the cells, directly or indirectly resulting in either beneficial or delirious effects in the plant. The sensitivity of the plant cell towards this radiation largely depends on the cell cycle and stage of cell division (Hafer *et al*., 2010; Pawlik and Keyomarsi, 2004). Gamma radiation can be either acute or chronic in terms of irradiation. Chronic irradiation speaks to longer exposure at lower dosages, whereas acute exposure refers to high dosages for a shorter period of time (usually one dose) (Mba *et al*., 2010). Chronic irradiation has been seen to produce fewer free radicals in wheat compared with acute radiation, while acute dosages increase chances of plant growth being affected negatively (Hong *et al*., 2018).

Gamma radiation also results in the formation of free radicals in cells due to interactions with molecules such as water (Nikogosyan and Angelov, 1981). They can be found in areas such as the cytoplasm of cells where they interfere with the cytochrome P450 and the mitochondrial respiratory chain (Pham-Huy *et al*., 2008). The high-water content in plant cells will further increase gamma-induced DNA damage, as more free radicals will cause more disruptions in the DNA structure, which will mostly be irreparable due to the low tempo of radiation (Caplin and Willey, 2018). As stated previously, seeds can be irradiated with higher dosages of gamma radiation in comparison to other tissue types such as callus which must be irradiated at lower levels.

Gamma radiation can also directly affect the integrity of DNA by creating genome mutations. This can be done mostly by DNA deletions and translocations such as terminal, reciprocal or interstitial chromosomal translocations (Szakács *et al*., 2010). Terminal translocations refers to a segment of a chromosome being replaced with another, reciprocal speaks to two chromosomes from different homologous chromosomes exchanging segments, while interstitial translocation refers to a piece of chromosome incorporated into another chromosome, usually without negative effects. These mutations have been detected in plants such as barley and black-eyed peas after gamma radiation (FAO, 2018; Farré *et al*., 2012). In a study done by Naito *et al*. (2004), gamma radiation induced very large DNA deletions, bigger than 6 Mbp in *Arabidopsis* pollen irradiated with 150 to 600 Gy rays. However, most of these deletions and translocations were not transmitted from the M1 generation to the progeny (Naito *et al*., 2004; Stadler and Roman, 1948). Chromosomal inversions due to gamma radiation can also occur, this refers to the detachment of a segment of chromosome which undergoes a 180° turn and then gets reattached to the same chromosome such as in sorghum. This experiment

was determined using 400 Gy irradiation and the changes in chromosomal arrangement were detected using electron microscopy (Mizuno *et al*., 2013). Segmental duplication has also been seen, but only applies to areas that have been duplicated and are larger than 1 kb in size. These duplications are added directly after the original segment that was used as the duplication base (Spencer-Lopes *et al*., 2018).

Other mutations that can also occur due to gamma radiation are missense mutations, nonsense mutations and silent mutations (Gulfishan *et al*., 2015). Missense mutations cause a changed triplet code to encode for a different amino acid. Nonsense mutation creates a premature stop codon due to a mutated triplet codon which results in a sudden stop in translation, thus affecting the protein transcription, while a silent mutation results in a mutation that has no effect in the amino acid codes and thus the translated proteins (Spencer-Lopes *et al*., 2018).

1.4.5 Assessment of damage done by mutagenesis

Quantitative assessment of M_1 damage and injury should be routine practise in mutation breeding programmes. Routine methods to assess M_1 injury is the determination of germination frequency and growth parameters (Zanzibar and Sudrajat, 2016). In addition, DNA damage caused by gamma irradiation can be assessed by the formation of micronuclei, which develop during irregular mitosis and serves as an indication of genomic damage caused by external sources (Shimizu, 2010). Micronuclei originate from chromosome damage that results in acentric chromosome fragments, as well as lagging chromosomes. This is due to a lack in functionality of the mitosis process where there is a severance in the chromatin bridge (Juchimiuk-Kwasniewska *et al*., 2011; Shimizu, 2010). Micronuclei formation usually occurs during anaphase and the micronuclei are then found in the cytoplasm.

The formation of micronuclei is in direct correlation to the dosage of the gamma irradiation, an increased gamma irradiation dosage will lead to increase formation of micronuclei (Vral *et al*., 2010). Micronuclei have been detected in mostly animal cells when exposed to gamma irradiation. For example, in mice bone marrow, high numbers of micronuclei formed when exposed to 10 Gy (Yalçin *et al*., 2010). In Chinese hamsters, fibroblasts exposed to 2 and 1.5 Gy resulted in 15% and 11% micronuclei formation, respectively, versus control cells showing no micronuclei (Zaichkina *et al*., 2004). In the fish, *Catla catla,* an increase of gamma radiation was connected to the increase in micronuclei formation (Anbumani and Mohankumar, 2012). However, micronuclei have also been detected in irradiated plant cells. For this, micronuclei formation was for example assessed, using FISH

(Fluorescence In Situ Hybridization), in barley meristematic cells where micronuclei formation was detected between ranges of 4.5% and 19.2%, depending on the dosage and rate of gamma irradiation (Juchimiuk-Kwasniewska *et al*., 2011).

Ultimately, molecular assays to recover all types of induced variations, such as SNPs, copy number variations and chromosomal rearrangements can be done by whole genome sequencing or approaches such as target induced local lesions in genomes (TILLING), random amplified polymorphic DNA markers (RAPD), or amplified fragment length polymorphisms (AFLPs) (Amri-Tiliouine *et al*., 2018; Suprasanna *et al*., 2015; Till *et al*., 2015). These techniques allow for the evaluation of the efficiency of mutation induction and identify target mutations in specific genes depending on the genome coverage of these screening techniques (Rutherford *et al*., 2014). Using RAPD markers, Khan *et al*. (2007) identified relatively similar sugarcane mutants after irradiation and Zambrano *et al.,* (2003) linked RAPD-derived polymorphic patterns to mosaic virus resistance. AFLPs were used to determine mutation frequency as this technique can be used to detect deletions and insertions which differ between two cultivars (Masiga and Turner, 2004). AFLP's have been used in barley to assess the differences in sequencing between 2 parental plants and their progeny (Castiglioni *et al*., 1998). AFLP's have also been used in chrysanthemum to detect differences between gamma radiated plants and non-radiated plants (Kang *et al*., 2013). TILLING, for example, allows for the early detection of point mutations after mutagenesis in genes of interest. For instance, Sato *et al*. (2006) used TILLING to detect small mutations induced in the M_2 generation of rice by gamma irradiation and the rate of mutation was estimated to be one per 6190 kb of the genome. Greene *et al*. (2003) did a study on EMS-induced mutations in *Arabidopsis* and detected on average 1 mutations per 170 kb using TILLING.

1.5 Technology used to increase the efficiency of mutation breeding

Any mutation breeding experiment requires the consideration of several parameters during project planning to ensure success. These can include:

1.5.1 Choice of explant

Various plant source material can be mutated in a mutation induction experiment. Seeds are the conventional tissue type and have been used as explant source many times in mutation breeding programs (Patade and Suprasanna, 2008). Seeds have the advantage that they can be desiccated, soaked, heated or even frozen prior to mutation induction. They can be stored for long periods and

are easy to handle and distribute. A second target source for mutation induction is pollen. The advantage of pollen mutation is that is allows for single cell selection procedures in combination with haploid systems while also removing chimerisms and includes the expression of alleles in the generation after induction (M_1) (Yang *et al*., 2004). The disadvantages of using pollen as source material, include difficulty in obtaining adequate amounts of pollen material and the fact that pollen's viability span is very short (Naito *et al*., 2004; Singson-Asuncion, 1988).

When the targeted crop species is vegetatively propagated, seedless-ness or production of limited seeds can be a problem, therefore other plant parts need to be considered for mutation induction experiments. *In vitro* tissue types that have been used for mutation breeding programs include root and shoot tips, cell suspensions, callus, leaflets, somatic embryos and meristematic tissue (Shu, 2012). Mutagenic treatment of these tissue types can be applied before, during or after the *in vitro* culturing of the tissue. The use of meristematic and callus as explant material for mutation induction is especially popular (Çelik and Atak, 2017). These cells or tissue types are toti- or pluripotent plant cells, likely rapidly dividing and actively involved in DNA replication, which are responsible for the formation of all cells needed in the life of adult plants (Gaillochet and Lohmann, 2015). Therefore, these mutations induced in meristem or callus cells are passed on mitotically to all derivative cells or tissues and mutations are soon "fixed" or lost in future cell generations (Klekowski *et al*., 1985). Additionally, *in vitro* tissue can rapidly be mass propagated before and after mutation induction. Also, both mutagenesis and screening of the M_1 population can be done *in vitro* (Saleem *et al*., 2005). The advantages of *in vitro* mutagenesis include the induction of mutations at high frequencies, even mutagen treatment since the mutagen can be assured to affect all cells equally and the use of selecting agents *in vitro* (Suprasanna *et al*., 2015). Lastly, using *in vitro* techniques saves space and time in handling large mutant populations and can be especially valuable in terms of keeping disease away from the plant material (Constantin, 1984; Patade and Suprasanna, 2008). However, *in vitro*-cultured cells and field grown plants can react very differently due to environmental influence on plant growth and development, especially with regards to abiotic and biotic stress conditions (Jan *et al*., 2018). This might lead to mutants selected or screened *in vitro* not transferring the desired phenotypes to *ex vitro* environments.

1.5.2 Selection for useful traits

A number of screening methods for selection of mutants with useful traits have been tested and are well established. These vary from visual methods of selection for traits such as disease resistance,

colour changes, plant growth and non-shattering of seeds, while mechanical methods for screening traits such as seed size and weight are also well established (Oladosu *et al*., 2015). Applying selection pressure for abiotic stress tolerance such as drought, heat or salinity, through laboratory experiments, hydroponic setups, field trials or *in vitro* systems are also well described (Lestari, 2006; Patade *et al*., 2006; Rai *et al*., 2011).

In the past *in vitro* selection has been used to successfully select genotypes that possess advantageous agronomic traits (Patade *et al*., 2008; Rai *et al*., 2011). *In vitro* selection specifically for osmotic tolerance that correlates to drought tolerance in the field has been done for crops like alfalfa (Dragiiska *et al*., 1996), sugarcane (Rao *et al*., 2013) and banana (Bidabadi *et al*., 2012). Chemicals such as PEG (polyethylene glycol), sorbitol and sucrose are routinely used as osmotica when added to *in vitro* nutrient growth media, used to maintain and grow plant tissue *in vitro*, and can mimic the effects of drought (Masoabi *et al*., 2017; Rai *et al*., 2011). These osmotica have been used in conjunction with plants such as rapeseeds and cereals (Channaoui *et al*., 2017). In addition, sucrose has the added benefit of acting as a carbohydrate source within the *in vitro* growth media, while a chemicals such as PEG, especially PEG with a high molecular weight, are non-toxic and are taken up by plant cells in limited quantities, depending on the plant species and exposed plant cell structure, these also lower the water potential of the plants (Blum, 2014; Rai *et al*., 2011). Thus, PEG can be used to mimic water stress in plants and such an *in vitro* selection system for osmotic tolerance in sugarcane callus has recently been successfully developed (Masoabi *et al*., 2017).

1.5.3 Mutagen choice

A first step in any mutagenic experiment is to establish the most appropriate mutagenic treatment. This usually involves the determination of the mutagen dosage for optimal mutation induction linked to normal vegetative growth, which typically involves a 50% lethality (LD50) or growth reduction (Oladosu *et al*., 2015). The LD50 value is dependent on the physiological condition of the explant tissue, the plant species, cultivar and genotype, as well as the type and dosage of mutagenic treatment applied. However, choosing dosages that lead to an LD50 might still result in desirable mutations being lost or overlooked due to plant mortality or poor agronomic performance in subsequent generations following the mutagenesis. It might therefore be more desirable to use lower LD rates to ensure the induction of new traits in already high-quality genetic backgrounds such as elite breeding lines. On the other hand, the choice of mutagen is dependent on the accessibility of the radiation source or

chemical, the preferable type of mutation to be introduced and the target tissue type, which will indicate the level of penetration needed.

1.6 Mutation breeding in sugarcane

1.6.1 Mutation breeding in sugarcane

Certain sugarcane germplasms possess traits that can successfully protect them or enhance their ability to resist adverse circumstances and these have been included in breeding programmes to development new cultivars throughout the years. However, to ensure sustainable agriculture of this crop, new ways for using available and induced genetic diversity must be developed.

It is well known that sugarcane has a large genome that has only very recently been sequenced and is still poorly annotated (Rutherford *et al*., 2014; Zhang *et al*., 2018). Modern commercial sugarcane derived from crosses of *Saccharum officinarum* and *Saccharum spontaneum,* an octa- and polyploid, respectively (Heinz, 1987). Over the years, sugarcane breeding programmes mostly relied on inter-crossing between hybrids, resulting in the current commercial sugarcane hybrids having mostly aneuploid genomes (Rutherford *et al*., 2015). Molecular diversity studies have revealed the limited genetic base of these commercial varieties and a need to diversify the genetic base (Nair *et al*., 1975). Spontaneous mutations, the primary source of all genetic variations in any organism, are very rare and occur randomly as a consequence of interactions with environmental factors (Kharkwal *et al*., 2012). However, these spontaneous mutations are too rare to provide sufficient variation needed in crop breeding programmes (Oladosu *et al*., 2015). Sugarcane also grows slowly, taking between 12 and 18 months to mature (Gentile *et al*., 2013), and produces limited seed, which has restricted the progress made with its breeding. Furthermore, sugarcane varieties with good quality traits are now showing signs of degenerating rapidly due to long-term cultivation (Khalil *et al*., 2018).

Mutation breeding has the potential to generate alterations in the genetic code of sugarcane, through chemical and physical mutagenesis, and utilise this genetic variability to develop sugarcane genotypes that can potentially survive especially abiotic stress (Oladosu *et al*., 2015; Pathirana, 2011). Mutagens can affect the genetic makeup of a plant indiscriminately, with no need for prior knowledge of the genome, and this method is potentially a viable option to generate suitable, well-adapted cane varieties containing genetic code not occurring naturally, resulting in useful traits.

1.6.2 Traits induced by mutation breeding

A review of the FAO/International Atomic Energy Agency's Mutant Varieties Database indicated that only 13 induced sugarcane mutant accessions had been registered in comparison to the 3000 registered mutants from over 170 plant species (FAO, 2018; Rutherford *et al*., 2013). These approved sugarcane mutant lines include traits such as resistance to red rot, adaptability, higher sucrose percentages and yield as well as resistance to *Ustilayo scitaminea* and improved quality of juice in sugarcane (FAO, 2018). Additionally, more recent research examples of mutagenesis applied in sugarcane include the use of chemical mutagenesis to produce sugarcane tolerant to herbicides such as imazapyr (Koch *et al*., 2012), plants resistant to sugarcane mosaic virus (SCMV) (Zambrano *et al*., 2003), selection and characterisation of sugarcane mutants with improved resistance to brown rust (Ali *et al*., 2007; Oloriz *et al.,* 2012; Sadat and Hoveize, 2012), as well as the application of physical mutagenesis to induce mutations *in vitro* in sugarcane for abiotic stress tolerance (Patade and Suprasanna, 2008; Nikam *et al*., 2015) and enhanced sucrose production (Mirajkar *et al*., 2016).

1.6.3 Mutagens used in sugarcane mutation breeding

Mutation breeding has been applied to sugarcane by either using chemical, such as EMS, or physical methods, such as gamma radiation or alpha particles (Rutherford *et al*., 2015). Ionising radiation has been used, gamma radiation specifically, to induce traits ranging from high sucrose and cane yield, to pest and disease resistance (FAO, 2018). Radiation dosage and exposure time for optimal mutation induction without negatively affecting overall plant performance has been extensively described. Depending on the explant type exposed to radiation, dosages range from as low as 10 Gy to 80 Gy gamma rays for sugarcane callus and vegetative cuttings (Ali *et al*., 2007; Khan *et al*., 2007; Nikam *et al*., 2008; Oloriz *et al*., 2011; Patade *et al*., 2008). From these studies it was established that 30 Gy to 50 Gy seemed to induce a lethal dose, where 50% of the tissue sample perished, depending on the development stage of the tissue.

The use of chemical mutagenesis to induce novel mutations in the complex genome of sugarcane has only recently advanced. EMS is one of the more regularly used chemicals as it is very effective in dividing cells due to an increase in the frequency of incorrect base pairing when DNA replication is active. It is also easy to use and provides a cost-effective way to produce novel mutations within plants (Pathirana, 2011). The ideal dosage for sugarcane was determined by several experiments indicating: 16 mM/4 hr EMS (Koch *et al*., 2012; Masoabi *et al*., 2017) or 0.1% to 0.5% EMS/30 min to 25 h (Khalil

et al., 2018; Purnamaningsih and Hutami, 2016) when using mainly callus as explant material. When a chemical such as sodium azide was used to induce mutations, concentrations of between 0.003% for 30 min (Oloriz *et al*., 2011), 1-5mg/L for 5 days (Mahmud *et al*., 2016) and 0.5% for 6 days (Ul-Haq *et al*., 2011) were used.

This study aims to induce novel mutations, through the use of gamma irradiation, and assess already available EMS-generated mutants, in a quest to generate sugarcane genotypes more tolerant to drought using mutation breeding.

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Chapter 2: RADIATION INDUCED MUTAGENESIS OF SUGARCANE CALLUS TO ENHANCE DROUGHT TOLERANCE

2.1 Introduction

Sugarcane is an important economic crop, both for its use in the sugar industry as well as its importance in biofuel production (Kang and Lee, 2015; Li and Yang, 2015; Solomon, 2014). However, sustainable sugarcane production is under threat due to climate change, where across the globe changes in average temperatures as well as levels of precipitation are a reality and likely to persist according to future predicted droughts (Lopes *et al*., 2011). South Africa specifically is currently experiencing irregular and declining patterns of rainfall and has seen temperatures increase annually in summer periods (Jury, 2013). Thus, even though sugarcane grows in warmer temperatures in tropical and sub-tropical regions, it requires large quantities of water, especially during the tillering and grand growth phases, for survival and optimal sugar production (Ferreira *et al*., 2017; Inman-Bamber and Smith, 2005; Sant'anna *et al*., 2013).

Sugarcane breeding for sustainable agriculture, with specific focus on drought tolerance, through traditional breeding methods faces numerous challenges. Sugarcane has one of the most complex, large and high ploidy genomes found in cultivated plant species, that so far has been poorly annotated (Gentile *et al*., 2015; Mancini *et al*., 2018). As a result, transfer of traits from donor to offspring is unpredictable due to chromosomes being randomly sorted in the genome (Bielig *et al*., 2003). In addition, sugarcane has a narrow gene pool and shows poor fertility (Lakshmanan *et al*., 2005). However, mutation breeding, through the use of gamma radiation, can produce stress-tolerant plants through the induction of novel mutations, using a variety of tissue types, irrespective of whether the genome is known or not, in existing sugarcane germplasm.

Gamma radiation is an effective and easy method of inducing mutations into plant cells due to its penetrative nature (Desouky *et al*., 2015). It is known to damage DNA structures, causing mostly double-stranded breaks, point mutations and small deletions (Morita *et al*., 2009). Gamma radiation also causes the formation of free radicals due to the ionisation of the water contained in the plant cells. These free radicals can cause damage to DNA as well as other structures in cells and can ultimately lead to cell death (Desouky *et al*., 2015). Radiation-induced *in vitro* mutagenesis has been successfully used in sugarcane for the introduction of mainly salt tolerance and sucrose accumulation (Mirajkar *et al*., 2016; Nikam *et al*., 2014; Patade and Suprasanna, 2008; Patade *et al.,* 2008).

The use of *in vitro* cells as target tissue for mutation induction is well described and includes tissue such as calli, plant cuttings as well as pollen samples (Beyaz and Yildiz, 2017). However, when aiming to introduce useful mutations with gamma radiation in these tissue types, it is important to determine the most efficient dose and exposure time, which will result in the maximum percentage of mutations without negatively affecting cell growth or cause cell death. In sugarcane several different dosages have been used to induce mutations and cause beneficial mutations. According to the FAO mutant variety database, 7 sugarcane cultivars have been registered using dosages of 30-150 Gy when irradiating tissue samples such as the bud of the sugarcane plant as well as seeds and seed stems (FAO, 2018). However, when Patade *et al*. (2008) irradiated embryogenic calli, lower dosages such as 10 Gy were used since this type of tissue was more fragile with high levels of cellular water. In addition, reported literature indicate the LD50 (lethal dosage, where 50% of the sample is lost) for gamma radiation in terms of sugarcane callus, as between 50-60 Gy (Çelik and Atak, 2017; Kaur and Gosal, 2008).

The present study aimed to use gamma radiation in an effort to induce beneficial mutations that can aid in enhancing drought tolerance in sugarcane in an effort to contribute towards the sustainable agriculture of this crop.

2.2 Material and methods

2.2.1. Plant material

Saccharum officinarum sp. hybrids NCo310 and N58 stalk tissue were harvested and washed with 96% ethanol. The leaves were removed aseptically in a laminar flow until the inner leaf roll tissues were exposed. The meristematic tissue was then cut into 2-3 mm thick discs and placed on semi-solid MS3 medium containing 4.4 g/l MS basal salts (Murashige and Skoog, 1962), 3 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid), 20 g/l sucrose, 0.5 g/l casein and 2.2 g/l gelrite, pH5.8, according to the methods described by Lakshmanan *et al*., (2005) and Snyman, (2004). Cultures were placed in a dark growth room at 26°C. After 6 weeks, which included sub-culturing onto fresh MS3 media every 2 weeks, callus was harvested and used for further experimentation.

2.2.2 Mutagenic treatment and in vitro osmotic selection of embryogenic callus

The efficiency of different radiation dosages to induce mutations without negatively effecting the viability of sugarcane callus was evaluated. For this callus clumps were harvested and 2.5 g of callus per culture plate was exposed to gamma radiation at dosages of 10, 20 and 40 Gy, four plates per treatment, at iThemba Labs, Western Cape, South Africa, with the assistance of Prof Kobus Slabbert. The ^{60}Co source provided an average dosage of between 3-4 Gy per minute. After radiation, following the method describe by Masoabi *et al*. (2017), callus was immediately transferred to fresh semi-solid MS3 media with the addition of 20% (w/v) PEG6000 (Sigma, USA) and kept in a dark growth room for 8 weeks, sub-culturing every 2 weeks onto fresh MS3-PEG media. Samples were then moved to a light growth room, at 26°C with a 16/8 hour light/dark cycle supplied by cool white fluorescence bulbs (Osram; 36W/640) with an intensity of 50 µMol photons/m^2/sec, after being placed on semi-solid MS0 media containing 4.4 g/l MS basal salts, 20 g/l sucrose, 0.5 g/l casein and 2.2 g/l gelrite, pH5.8, without PEG6000, for 2 weeks to recovery from the osmotic stress. Initially, for the first week in the light growth room the callus cultures were covered with a thin yellow cellophane filter layer to reduce the light intensity. After two weeks, the *in vitro* tissue was placed on semi-solid MS0 growth medium containing 20% (w/v) PEG6000 for 6 weeks, with sub-culturing taking place every 2 weeks, to allow for the germination of somatic embryos. Control samples were not exposed to radiation but cultured on PEG6000. Surviving plantlets were transferred to Magenta culture jars containing semi-solid MS0 media and allowed to grow to a height of around 5 cm. Plantlets were then hardened off in the glasshouse by planting them in a soil mix (2:1:1 potting soil: sand: vermiculite), with pots being covered with transparent plastic containers to prevent wilting for 2 weeks. Plants were kept under natural light at around 25°C in the glasshouse.

Callus growth, embryogenicity and cell death of the calli, irradiated at different dosages, were compared with the control calli, eight weeks after exposure to the gamma source. Furthermore, somatic embryo formation, plantlet regeneration, abnormal shoot development (chlorophyll deficiency of regenerated shoots), and rooting of plantlets were monitored from tissue exposed to the different irradiation treatments.

2.2.3 Statistical analysis

For statistical analyses of callus growth and somatic embryo development across all radiation dosages, four sample replicates (four culture plates of 2.5 g callus) were included in the experiment. Relative callus growth was quantified by measuring the callus cover area (mm^2) using an isometric grid system.

Significant difference, p value ≤ 0.05, for the total callus coverage of each plate for the different dosages were calculated using a one-way ANOVA and a Bonferroni *post hoc* test.

2.3 Results

2.3.1 Effect of irradiation and osmotic selection on callus growth

N58 and NCo310 callus, 2.5 g per plate, were irradiated with 10, 20 or 40 Gy gamma rays and placed immediately on *in vitro* PEG selection media for 8 weeks (Figure 2.1). After the 8-week incubation period, callus clumps showed signs of cell necrosis and a reduction in callus growth, linked to irradiation dosages, when compared to non-irradiated control callus (Figure 2.2). N58 control callus not exposed to radiation, showed high levels of cell necrosis, indicated by dark brown callus clumps, after 8 weeks of osmotic selection in the dark, similar to callus exposed to 20 or 40 Gy. All calli exposed to 10 Gy were lost due to contamination. During the 8-week callus selection phase, the relative amount of the N58 control callus stayed almost constant while callus exposed to 20 and 40 Gy decreased slightly by 1.04 and 1.38-fold, respectively (Table 2.1).

NCo310 control callus turned a dormant pale brownish colour after 8 weeks on osmotic selection medium, while calli exposed to 20 and 40 Gy stayed a healthy cream colour (Figure 2.2). Almost no cell necrosis (dark brown cell clumps) were visible in the callus of all the different treatments, including the control callus. Control calli growth decrease on average 1.56-fold, while the amount of the irradiated calli stayed constant (20 Gy treatment) or decreased only slightly, 1.2- and 1.1-fold decrease, respectively for the 10 and 40 Gy treatments (Table 2.1).

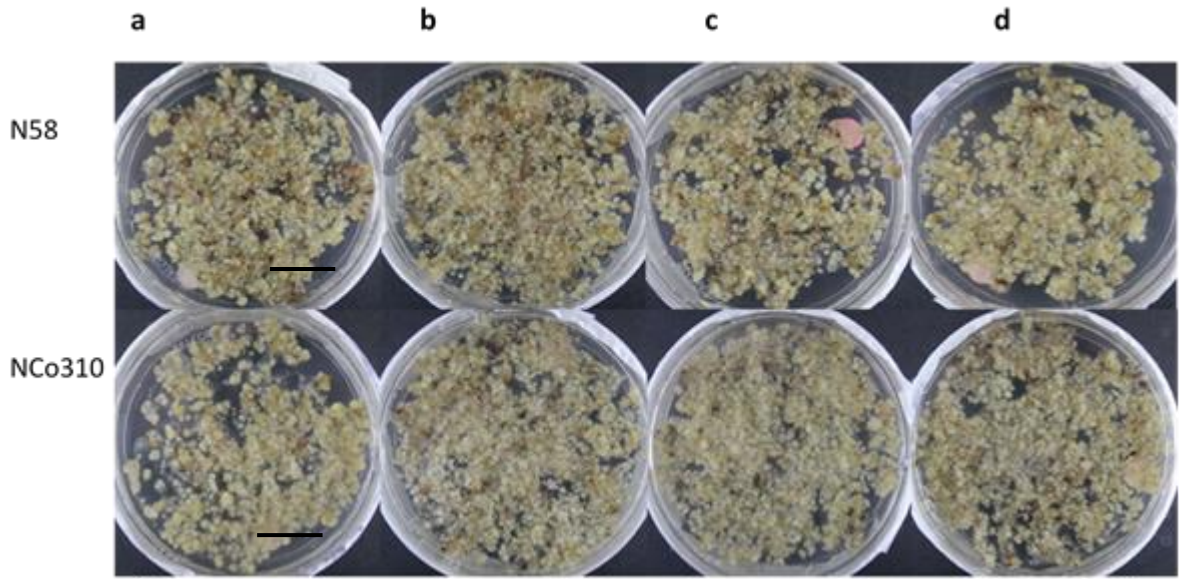

Figure 2.1: N58 and NCo310 sugarcane callus immediately after irradiation with different gamma ray dosages namely, **a)** 0 Gy, **b)** 10 Gy, **c)** 20 Gy or **d)** 40 Gy. Size bar represents 2 cm.

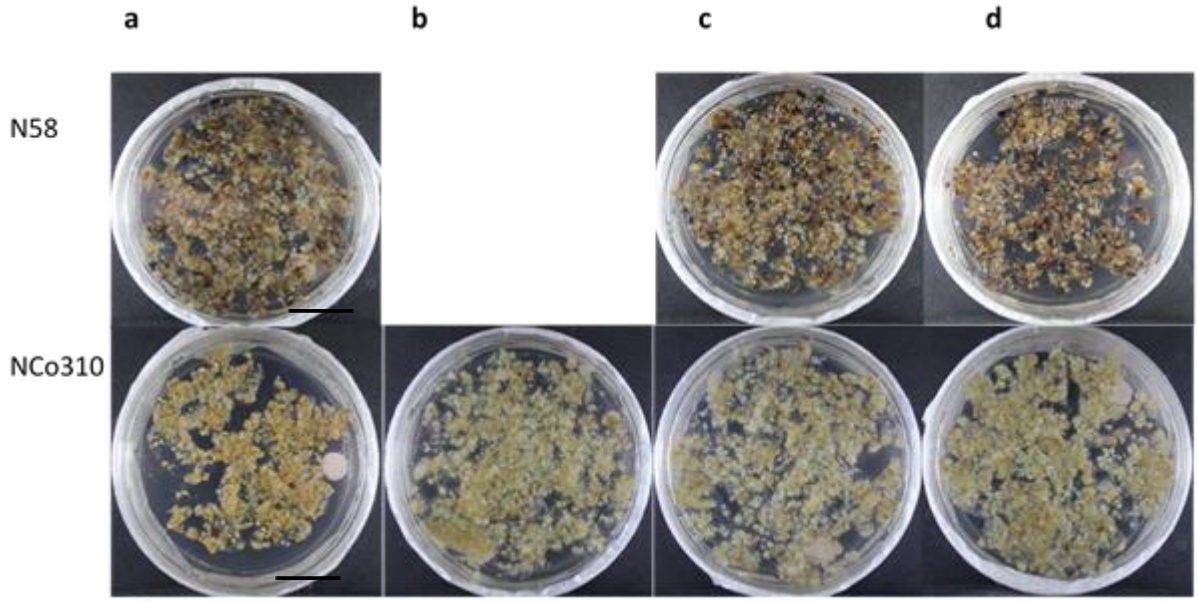

Figure 2.2: N58 and NCo310 sugarcane callus after 8 weeks in the dark, on semi-solid MS3 containing PEG6000, after irradiation with different gamma ray dosages, **a)** 0 Gy, **b)** 10 Gy, **c)** 20 Gy or **d)** 40 Gy. Size bar represents 2 cm.

Table 2.1: Growth of N58 and NCo310 callus (mm^2 coverage of plate area), on semi-solid MS3 media containing PEG6000, after exposure to different radiation dosages (10, 20 and 40 Gy) and culturing for 8 weeks in the dark at 25°C. Significant difference at $p \leq 0.05$ is indicated with an (*) and was determined using a one-way ANOVA followed by a Bonferroni *post hoc* test.

CULTIVAR	IRRADIATION DOSAGE (GY)	CALLUS COVERAGE (MM^2)
N58	0	26 ± 0.07
	20	25 ± 0.82
	40	18 ± 0.69*
NCO310	0	15 ± 1.83
	10	22 ± 1.83*
	20	26 ± 1.82*
	40	24 ± 1.35*

2.3.2 Effect of irradiation and osmotic stress on somatic embryo development

The callus regeneration and selection phase for N58 and NCo310, were followed by the transfer of the callus cultures to MS0 medium containing PEG for the formation of somatic embryos. When the N58 cultures were incubated in the light growth room for 6 weeks, under osmotic selection pressure, somatic embryos, indicated by small green growth points, started to germinate (Figure 2.3 and 2.4). The number of these embryos were recorded. On average N58 callus radiated with 20 Gy and 40 Gy gamma rays formed 11 and 4 somatic embryos per culture plate respectively, compared to the 6 formed on non-irradiated control culture plates (Figure 2.4). For the NCo310 cultivar, callus mostly showed signs of cell death seen as dark brown callus clumps (Figure 2.3). A number of somatic embryos emerged during the first few weeks in the light growth room on MS0. However, these embryos showed signs of chlorosis and soon died. From control callus, not exposed to radiation, no somatic embryos germinated as the callus turned black, indicating necrosis and cell death (Supplementary data, Figure 5.3 and 5.4).

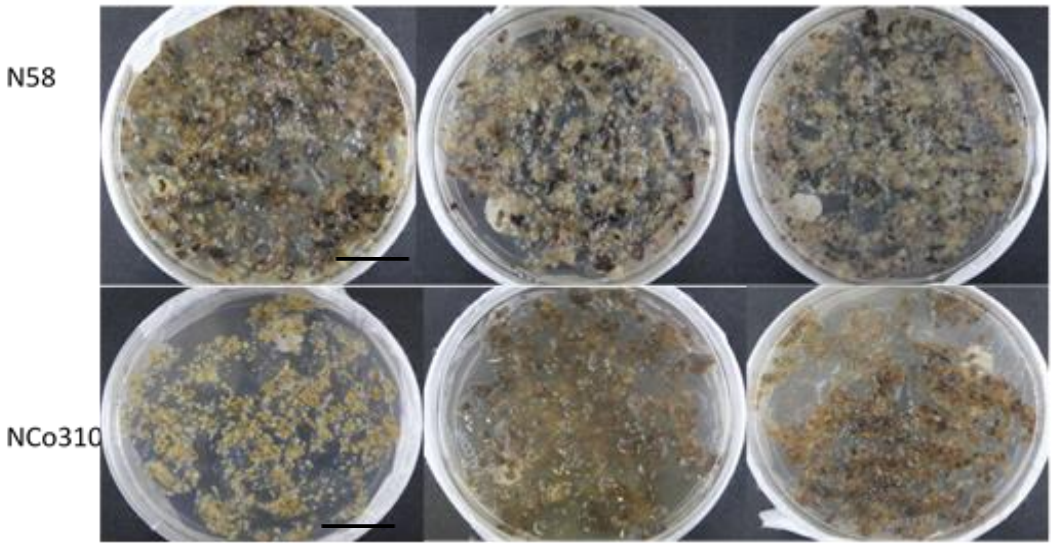

Figure 2.3: N58 and NCo310 callus cultured for 6 weeks in the light growth room on semi-solid MS0 medium containing 20% PEG6000 after initial exposure to different gamma ray dosages, **a)** 0 Gy **b)** 20 Gy or **c)** 40 Gy. Size bar indicates 2 cm.

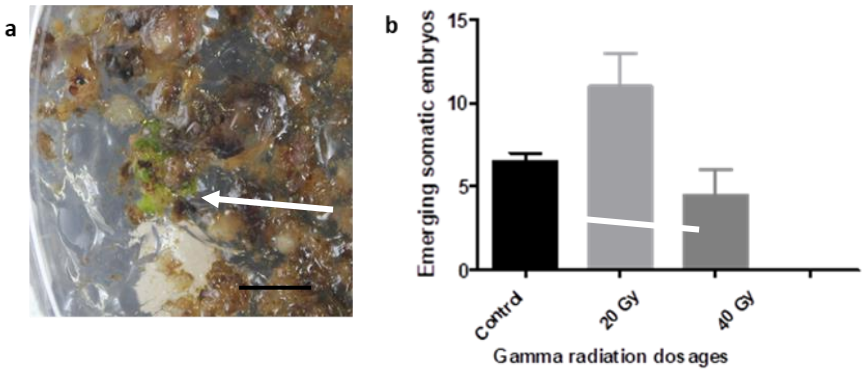

Figure 2.4: Somatic embryo formation from irradiated N58 callus cultured after 6 weeks in a light growth room on semi-solid MS0 medium containing 20% PEG6000 initially exposure to different gamma ray dosages (0, 20 or 40 Gy). **a)** Somatic embryos emerging from callus exposed to 20 Gy; **b)** Mean number of somatic embryos that developed per culture plate exposed to each of the different gamma ray dosages, (n = 4). No significant difference was seen. Size bar indicates 0.5 cm.

2.3.3 Plantlet regeneration

Emerging somatic embryos were allowed to mature for a further 4 weeks on PEG selection growth media (Figure 2.4). However, for the N58 cultivar only three plantlets in total for the whole experiment (4 culture plates each containing 2.5 g of callus, irradiated at three different dosages), two and one plantlets, for 20 and 40 Gy treatments respectively, survived the strenuous osmotic selection process (Figure 2.5). The somatic embryos that had developed from the control treatment all stopped

developing and showed overall necrosis. The three plantlets were allowed to mature further, allowing time for shoot elongation and root development. Nevertheless, only one plantlet from the initial 20 Gy treatment was able to form roots and survive on semi-solid MS0 growth media containing PEG (Figure 2.5b). NCo310 samples, had by this time, all succumbed to cell necrosis and further experimentation concerning them were impossible.

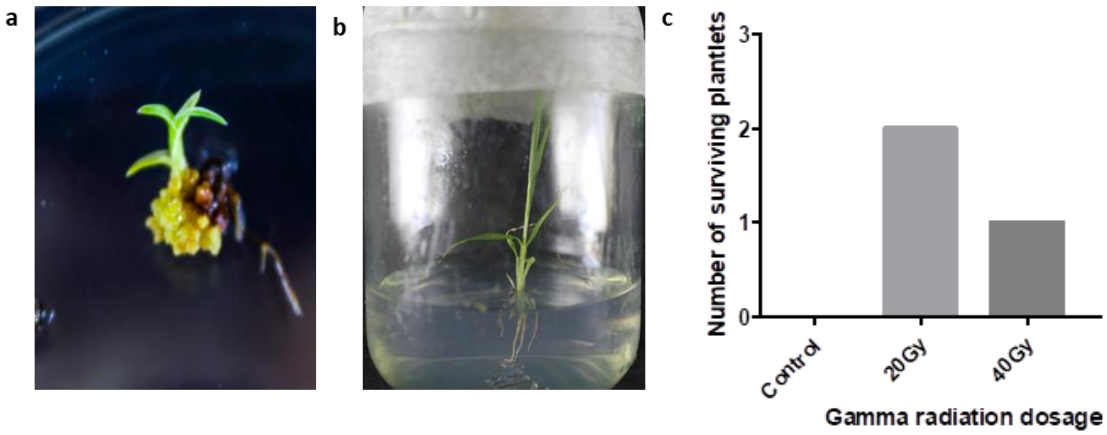

Figure 2.5: N58 plantlet development after gamma radiation exposure and *in vitro* osmotic selection on 20% PEG6000. **a)** Maturing somatic embryo after 16 weeks; and **b)** plantlets formed after 20 weeks in light growth conditions; **c)** total number of surviving plantlets after radiation exposure to gamma dosages between 10 and 40 Gy.

2.4 Discussion

Gamma radiation is a well-known method of inducing random mutations in the genome of plants for crop improvement, specifically targeting traits to withstand adverse environmental conditions such as increased temperature and drought (FAO, 2018). This study, although not using a novel approach, was aimed at potentially developing novel sugarcane genotypes with enhanced drought tolerance in the NCo310 and N58 cultivars. NCo310 and N58 are both important commercial sugarcane cultivars (Nuss and Brett, 1995; Zhou, 2013), but conflicting reports exist on the level of drought tolerance these cultivars display (Fawaz *et al*., 2013; Inman-Bamber, 1982; Zhang *et al*., 1996).

In the past various different types of tissue, used as explant material, have been radiated in mutation breeding experiments. Tissues that are routinely irradiated include callus, cuttings, seed and pollen (Beyaz and Yildiz, 2017). Based on known research, when callus is irradiated, growth alterations linked to dosage increase can occur. These include reduced growth and cell differentiation, which are due to

either direct damage caused by the radiation to the DNA or indirectly through the formation of reactive oxygen species (ROS), which can also damage DNA and other cellular structures, compromising growth and development (Desouky *et al*., 2015). For example, a study involving Barberton daisies, gamma irradiation of callus reduced the biomass of the callus significantly and the regenerated plants were stunted compared to the non-irradiated control plants (Hasbullah *et al*., 2012). Indica rice callus exposed to increase gamma dosages resulted in a decrease in survivability (Hossain and Alam, 2001). Sugarcane callus growth and proliferation of embryogenic callus declined, while cell necrosis increased with increase gamma radiation dosages (Moallem *et al*., 2013; Nikam *et al*., 2014; Patade *et al.,* 2008). Thus, gamma rays seem to affect cell growth and survival in callus across different plant species.

In mutation breeding experiments the frequency of the induced mutations are a direct result of the dosage and rate of exposure to the mutagen. It is generally assumed that a high dose does not necessarily yield the best results (Oladosu *et al*., 2016). A balance should be found between the mutation load, tissue survival and the chance to find desirable mutations. It has been suggested that a lower mutagen dosage, while resulting in lower mutation rates and thus in less severe mutations, could enable enhanced growth and development (Kim *et al*., 2000). In the past, when the mutation of sugarcane using gamma radiation was attempted, dosages varied over a large range, from as little as 10 Gy to as much as 100 kGy, depending on the explant type that was irradiated (Kwon-Ndung and Ifenkwe, 2000; Nikam *et al*., 2014; Patade and Suprasanna, 2008; Patade *et al*., 2008). For example, one-budded sugarcane setts were irradiated at optimal dosages linked to specific genotypes of between 5 to 8 kGy (Kwon-Ndung and Ifenkwe, 2000), while vegetative cuttings were irradiated at 10 to 40 Gy (Khan *et al*., 2007). However, when sugarcane callus was used as explant material for radiation experiments, dosages of between 10 to 80 Gy were used. Optimal dosages were set at between 10 and 30 Gy, with dosages > 40 Gy resulting in limited *in vitro* tissue totipotency and regeneration (Nikam *et al*., 2014; Patade *et al*., 2008). In this study, sugarcane callus from the N58 and NCo310 sugarcane cultivars were generated, harvested and gamma irradiated at dosages varying between 10 Gy and 40 Gy. The callus exposed to gamma rays then underwent *in vitro* osmotic selection on PEG6000 according to the method established by Masoabi *et al.* (2017), which identified the ideal PEG concentration leading to cell death but allowing growth and development of sugarcane calli displaying an increased osmotic stress tolerance phenotype with putative tolerance towards drought.

Irradiated N58 callus, selected on PEG, showed a decrease in growth linear with an increase in gamma dosage, while irradiated NCo310 callus growth was not affected (Figure 2.1 and 2.2). However, non-radiated NCo310 callus placed on PEG selection showed a significant decrease in growth not seen in

the N58 control callus. The osmotic selection method described by Masoabi *et al*., (2017) was specifically developed for callus of the NCo310 cultivar, allowing for very limited or no survival of callus cells on the PEG6000 selection regime also seen in this study. With prolonged osmotic stress exposure, N58 callus, both irradiated and non-radiated, retained their opaque/white colour with a few brown callus clumps indicating cell necrosis at percentages of 60%, 50% and 60% for the control, 20 Gy and 40 Gy samples, respectively (Figure 2.2).

After the callus growth and selection phase, a limited number of N58 somatic embryos germinated from callus exposed to 20 Gy and 40 Gy of which only 1 plantlet, radiated at 20 Gy (1 plantlet/1 g calli), was able to mature and form roots (Figure 2.4 and 2.5). In contrast to the irradiated N58 samples, the NCo310 callus exposed to gamma radiations and under osmotic selection pressure was unable to form any maturing somatic embryos and no plantlets developed from this treated tissue samples (Figure 2.2 and 2.3). In other reported studies, 7, 18 and 19 plantlets were regenerated from three sugarcane cultivars, namely NCo376, N19 and NCo310, after chemical mutagenesis of 1 g callus and osmotic selection (Masoabi *et al.* 2017). Khalil *et al.* (2018) worked with two sugarcane cultivars, FN39 and ROC22, and regenerated 2 and 18 plantlets respectively from 100 embryogenic calli pieces, after mutation induction with EMS and osmotic selection with PEG. Munsamy *et al.* (2013) used no selection in their experimentation, but the chemical azide was used to induce mutations in sugarcane callus which resulted in 12 plantlets per 0.2 g of treated callus. Oloriz *et al.* (2011) regenerated 5 plantlets after physical and chemical mutagenesis, and came to the conclusion that the chemical NaN3 resulted in higher selection frequency for plantlets that showed resistance to brown rust. Lastly, Nikam *et al.* (2014) regenerated a large number of sugarcane plantlets (138) after gamma radiation and selection for salt tolerance. However, it is unclear what amount of callus was initially irradiated but the study reported that an increase in radiation dosage coupled with selection pressure significantly reduced the number of surviving plantlets. In addition, Patade *et al*. (2008) exposed CoC-671 sugarcane calli to gamma radiation and observed a severe drop in plantlet regeneration from control non-irradiated callus to irradiated callus subjected to salt selection, which generated an average of 6 plantlet or 1 plantlet per 200 mg of treated callus, respectively. To summarise, it seems from these reported results and the current study that the type/dosage of mutagenic agent, relevant cultivar and the selection pressure applied can all influence the frequency of mutagenic plantlet regeneration.

In the future, a drought trial will have to be conducted with the identified N58 putative drought tolerant mutant clone. Data such as relative leaf water content, stomatal conductance, photosynthesis rate, root architecture and water potential will give an indication of the level of drought tolerance existing in this mutant plant compared to the normal N58 cultivar. Also, this mutant plant will have to be assessed under non-stress environmental conditions to confirm normal growth phenotypes,

structure and sucrose levels. In the long term, field trials will need to be conducted to confirm drought tolerance in a natural environment.

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Chapter 3: HISTOLOGICAL ASSESSMENT OF RADIATION INDUCED DAMAGE IN SUGARCANE TISSUE THROUGH THE DETECTION OF MICRONUCLEI

3.1 Introduction

Radiation can cause direct damage to DNA. This damage caused to DNA in higher plants can result in many different trait adaptations, some of interest to agriculture, such as changes in tiller length, seed germination, flower growth, plant size and yield as well as changes in tolerance to biotic and abiotic stresses (Jan *et al*., 2011; Marcu *et al*., 2013; Songsri *et al*., 2011). Alterations in DNA during mutagenesis can include intragenic point mutations within a gene sequence, intergenic sequence duplications, translocations and deletions and also changes in chromosome structure and numbers (Oladosu *et al*., 2015).

Micronuclei are chromosome fragments or whole chromosomes which are left behind at anaphase during cell division. These micronuclei are then covered in a nuclear membrane and are not included in the main nucleus during telophase (Luzhna *et al*., 2013). These micronuclei can be seen close to the nucleus and can be identified by their nucleus-like structure, although they appear much smaller than the correctly formed nuclei. They originate after extensive DNA damage and are considered to be biomarkers of genotoxicity that can be correlated with DNA double-strand breaks and DNA recombination incidents (Heddle *et al*., 1983).

The induction of micronuclei following radiation exposure is well known, and it is predicted, according to the literature, that more chromosomal damage will occur and thus more micronuclei will form, at higher dosages of radiation (Aypar *et al*., 2011; Balajee *et al*., 2014; Luzhna., *et al.*, 2013; Streffer *et al.*, 2018;). In this study, sugarcane callus was irradiated with different dosages of gamma radiation. To assess the DNA damage at the various dosages, the formation of micronuclei in these irradiated calli was investigated.

In sugarcane, limited information on protocols for callus histology are available. In the past, most histological sugarcane studies focussed mainly on microscopy of lignin and cell wall structures in relation to biofuel production, cell structures when infected with pathogens, chromosome and genomic structure of interspecific hybrids and *in vitro* embryogenic callus structures (Coletta *et al*., 2013; de Alcantara *et al*., 2014; de Souza *et al*., 2012; Fontaniella *et al*., 2002; Marques *et al*., 2018; Rodríguez *et al*., 1996; Sant'Anna *et al*., 2013; Vieira *et al*., 2018; Yu *et al*., 2018). However, to our

knowledge, no information is currently available on the histological assessment of DNA damage, including micronucleus formation, in sugarcane.

A number of methods for the fluorescent labelling and imaging of DNA and chromosomes have been described. Staining of nucleic acids in plants include compounds such as Hoescht, haematoxylin and eosin and DAPI (4',6-diamidino-2-phenylindole) (Fischer *et al*., 2008; Kapuscinski, 1995; Laloue *et al*., 1980). However, specifically in plants, histological techniques to detect stains and fluorescent dyes bound to DNA have several potential challenges. These include permeability of plant cell walls resulting in low labelling efficiency, interference with DNA structure and endogenous auto-fluorescence due to mainly chlorophyll (Roshchina, 2012). Thus, in this chapter we focussed on the testing and establishment of techniques that could be used to optimally detect nucleic acids in sugarcane cells, with specific focus on the detection of micronuclei as a marker for gamma radiation-induced DNA damage.

3.2 Material and methods

3.2.1 Plant material and establishment of in vitro cultures

Saccharum officinarum sp. hybrid NCo310*,* stalk tissue was harvested and washed with 96% ethanol after which the mature leaves were removed aseptically. The apical meristem of the exposed leaf rolls were cut into 2 mm thick discs and placed on MSC3 medium containing 4.4 g/l MS basal salts (Murashige and Skoog, 1962), 3 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid), 20 g/l sucrose, 0.5 g/l casein and 2.2 g/l gelrite, pH 5.8. Cultures were placed in a dark growth room at 26°C. After 5 days of incubation, these discs were exposed to different dosages of gamma radiation, or the discs were cultured for 6 weeks, sub-cultured onto fresh MSC3 media every 2 weeks, until callus formed. Callus clumps were then harvested and exposed to gamma radiation (Figure 3.1). For all experiments callus was used while only a few included the use of discs.

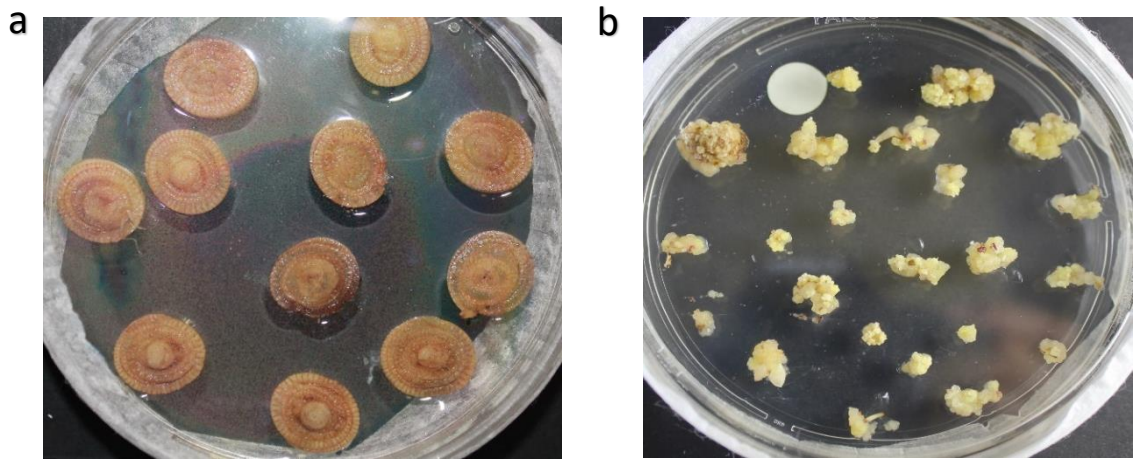

Figure 3.1: Sugarcane *in vitro* tissues exposed to irradiation, including images of **a)** leaf discs 5 days after and **b)** callus 6 weeks after culture initiation.

3.2.2 Irradiation of plant material

Tissue samples, callus or discs, were placed on new MSC3 media, 2.5 g of callus or 10 discs, per culture plate. Samples were then exposed to 10 Gy, 20 Gy or 40 Gy, at iThemba Labs, under supervision of Prof Kobus Slabbert (Radiation Biophysics Group), while control samples were not exposed to radiation. After radiation, callus and discs were kept on the same media and transferred to the dark growth room for 12, 24 or 36 hours. Samples were collected at these time points and processed for the use in different microscopy techniques, as stated below, to assess micronuclei formation.

3.2.3 Fluorescent and light microscopy

Tissue fixation and dehydration

Method 1

Irradiated samples were fixed overnight in a 3:1 solution (v/v) (enough to cover the samples) of 100% ethanol and 17.4 M glacial acetic acid (Merck, USA), using the method described by Yeung *et al*. (2015). Samples were then rinsed three times with water for 15 min followed by hydrolysis in hydrochloric acid (5 N) at room temperature for 60 min. After hydrolysis the samples were again rinsed with water, three times for 5 min each.

Method 2

Non-diluted isopentane, 100 ml, (Merck, Germany) was cooled in liquid nitrogen. Tissue samples were placed on a cork tile and covered with tissue freezing media (Leica Biosystems, RSA) and submerged

in cold isopentane until the tissue freezing medium became white/opaque in colour. Samples were stored at -80°C. A Leica Cryostat (Leica CM1860 UV, Leica Biosystems, RSA) was used to make 40 µm sections of the frozen tissue samples at -20°C. Cut tissue sections were stored at 4°C. This method was optimised by the Central Analytic Facility (CAF) at Stellenbosch University in terms of their freezing protocol.

Method 3

Tissue samples were fixed overnight in a 3:1 ratio (v/v) of 100% ethanol to 17.4 M glacial acetic acid solution and hydrolysed in 5 N hydrochloric acid, as described in Method 1, followed by dehydration in 100% ethanol. Samples were placed in a 1:1 ratio of ethanol:LR white resin (v/v) (Polyscience Inc, London Resin company LTD) followed by immersion in 100% LR white (Braselton *et al*., 1996). Samples were then placed in capsules (Agar Scientific, UK) containing the LR white resin and placed at 60°C until samples solidified. The capsules were then cut and sectioned, between 500 to 1000 nm, with an Ultramicrotome (Leica UC7 Ultramicrotome, Germany).

Method 4

Tissue samples were fixed in 10% formal saline (Merck, USA) for 7 days, followed by dehydration protocols with 70%-100% ethanol for 2 hours. Afterwards samples underwent clearing (the addition of a miscible agent after the dehydration process) with xylene (Sigma Aldrich, USA), and finally saturation within paraffin wax (enough to cover the sample). Dehydrated tissue was embedded using a TISSUE TEK II, model 4640B automated tissue processor (Lab-Tek division, Miles Laboratories Inc, Naperville, IL). Samples were cut in 4 µm sections with a rotary microtome (Reichert Jung, Heidelberg, Austria). The process of paraffin embedding was optimised by the CAF at Stellenbosch University.

Tissue staining

Tissue samples fixed with ethanol:acetic acid were stained in Schiff's reagent (Sigma, USA) for 3 hours at room temperature, followed by toluidine blue (Merck, USA) staining, as per the Feulgen method described by Braselton *et al.*, (1996). Schiff's reagent stains cell walls deep purple, while toluidine blue has a high affinity for acidic cell compounds, thus it stains DNA a dark blue colour. After the allotted staining time (2-3 hours), the samples were washed three times in cool water (4°C), 10 min per wash. The sugarcane samples were dehydrated by the addition of 100% ethanol, which was replaced three times after 10 min. Samples were stored in 100% ethanol until used. Tissues fixated with isopentane were stained with 20 mM propidium iodide (PI; Life Technologies, USA) for 10 min at room temperature.

Callus tissue fixed with formal saline were stained with one of several stains, including eosin (Sigma-Aldrich, USA), haematoxylin (Sigma-Aldrich, USA), or Hoechst 33342 (Invitrogen, H3570, USA) containing trihydrochloride and trihydrate (1 to 8000 phosphate buffered saline (PBS)). Staining was done for 45 min in the dark. Eosin and haematoxylin stained tissue were de-paraffinized (xylene dips were used for 10 min) and rehydrated (ethanol 100% to 70%) following staining and stored at room temperature for further use. After staining with Hoechst, tissue was washed in PBS superseded by dehydration for about 10 sec for each concentration (70%-100% ethanol) after which the sample was allowed to dry. Fluorescent mounting media (Dako, Denmark) was used to add a cover slip and slides were stored at -20°C until all samples were ready for imaging.

Sugarcane callus was placed on a microscope slide, after which DAPI (ThermoFischer, ZA) staining was added on top, followed by washing after 10 min of staining. Callus was then visualised through fluorescence microscopy.

Enzymatic tissue digestion and staining

Method 5

Clumps of callus cells were placed in 50 ml Erlenmeyer flasks containing 20 ml of liquid MSC3 media and 1% pectinase (Sigma, USA) to facilitate the breakup of cell clumps into single cells in suspension (Ranabhatt and Kapor, 2018). Samples were incubated at room temperature in the dark on a shaking incubator for 12 or 24 hours. Cell suspensions were stained using PI (propidium iodide) 9 (Life Technologies, ZA) or SYTO 9 (Life Technologies, ZA) for 20 minutes at their full concentrations.

Tissue imaging

For the imaging, excluding DAPI and H&E (Hematoxylin and eosin) staining, the LSM780 Elyra PS1 confocal microscope (Zeiss, Germany; Central Analytic Facility, Stellenbosch University) was utilised in combination with the ZEN 2012 z-stacks software to process and display images at maximum intensity projections. The objective EC Plan-Neofluar 10X/0,30 was used with z-stacks at 5 µm increments and a path detector with a 32 GaAsP channel to gather light.

For DAPI and H&E staining, a Nikon ECLIPSE E400 biological microscope, was used and a digital colour camera (Nikon DXM1200) 40x was utilised to capture images both for bright field and fluorescence imaging.

For fluorescence imaging of Hoechst, SYTO 9 and PI, the 405 nm, 488 nm and 561 nm lasers were used respectively for excitation, while emission was detected in the ranges 410-475 nm, 481-579 nm and 584-735 nm, respectively.

3.2.4 Scanning electron microscopy (SEM)

Tissue fixation and dehydration

Tissue samples were fixed overnight in a 3:1 solution (v/v) of 100% ethanol and glacial acetic acid (Merck, USA). Following the overnight fixation process, samples were washed 3 times in water for 15 min and submerged in 2 ml of (v/v) hexamethyldisilazane (HMDS; Sigma-Aldrich, USA) and ethanol, at a 1:2 ratio, for 20 min. This was followed by firstly, the addition of a 2:1 HDMS:ethanol solution, incubation for 20 min, and secondly, by a 100% HDMS solution, incubation for 20 min, and repeated twice (volumes were dependant on the tissue, and the solutions were added until the samples were submerged). Finally, the samples were placed in a fume hood overnight, allowing the HDMS to evaporate, after which the samples were stored in a desiccator until used.

Sugarcane tissue samples were cut into sections, using a scalpel, and fixed overnight in a 0.1 M phosphate buffer, pH7.4 containing 2.5% (v/v) glutaraldehyde (25% EM grade, Agar Scientific, UK). Tissues were then placed for 1 hour in a 1% osmiumtetroxide solution (4%; SPI Supplies, USA). Samples were dehydrated in a series of ethanol concentrations (30%, 50%, 70%, 90% and 100%) for 10 min each until drying with 100% HMDS.

Mounting was done on aluminium SEM stubs (Agar Scientific, UK) using double sided conductive carbon tape (SPI, USA), which was cut into sections and used to attach the dried samples to the stubs. Samples were sputter-coated with a layer of gold using an Edwards S150A sputter-coater.

Tissue imaging

Tissues samples were visualised on a Zeiss Merlin Field Emission Scanning Electron Microscope (FESEM) (Carl Zeiss Microscopy, Germany), situated at the CAF, Stellenbosch University, in combination with secondary electron (SE) detection at an accelerating voltage of 5 kV, a probe current of 250 pA (picoampere) and at a working distance of 3 to 4 mm.

3.2.5 Scanning Transmission Electron Microscope (STEM)

Sugarcane tissue samples followed the SEM preparation protocol described above. However, after dehydration, samples were gradually infiltrated, with 25%, 50%, 75% and 100% Spurr's Embedding Resin 100% (enough to cover the samples; Spurr, 1969) (Sigma Aldrich, USA), followed by

polymerisation at 60°C for 48 hours. Thin sections (70 nm-100 nm) were made of the resin samples using the Leica Ultramicrotome, which were then collected on 200 mesh copper grids and stained with 2% uranyl acetate (SPI, USA) for 10 min and Reynold's Lead citrate (RLC; Sigma Aldrich, USA) for 2 min. The same microscope used in SEM imaging was used for visualisation, however, a STEM detector was used at an accelerated voltage of 20 kV and a probe current of 250 pA.

3.2.6 Summary of methods used

Table 3.1. Summary of steps followed in tissue preparation for the histological study of sugarcane genetic cell material.

METHOD	FIXATION	HYDROLYSIS	DEHYDRATE	EMBEDDING	SECTIONING	STAINING	DETECTION
1	Ethanol/ Acetic acid	HCl			Hand sectioning	Schiff (Feulgen)/ Toluidine blue	Fluorescent
2	Isopentane/ Freezing media			Embedded in tissue freezing media	Cryostat (40 µm)	PI and SYTO9	Fluorescent
3	Ethanol/ Acetic acid	HCl	Ethanol	LR white	Microtome (500 nm)	Schiff (Feulgen)/ Toluidine blue	Light
4	Formal saline		Ethanol + Xylene	Paraffin wax	Microtome (4 µm)	H&E, Hoechst, DAPI	Light (H&E), Fluorescent (Hoechst, DAPI)
5	(Pectinase treatment)				No sectioning	PI, SYTO 9	Fluorescent
SEM	Ethanol/ Acetic acid		Ethanol/ HMDS		Aluminium stubs	Sputter coated	FESEM*
STEM	Ethanol/ Acetic acid		Ethanol/ HMDS	Spurr's resin	Ultramicrotome (70 nm)	Uranyl acetate / RLC	FESEM* + detector

**FESEM: Field Emission Scanning Electron Microscope*

3.2.7 Data analysis and statistics

All quantitative measurements were made with three biological repeats with three technical repeats each. A culture plate containing 2.5 g callus or 10 leaf discs were considered a biological sample, while technical repeats consisted of a microscope slide made from tissue collected from a biological sample.

Images of each time point for each radiation dosage were used to quantify micronuclei results. Three technical repeats were used for each time period and dosage and a 4x4 block was used to count the number of micronuclei seen in each image. For each image, 3 different biological repeats were used so as to create diversity as some cells might have more micronuclei present than others.

Mean values are presented with their standard deviations (SD). All graphics and statistically analysed were conducted using Graphpad Prism version 6.01 for Windows (GraphPad Software, La Jolla, USA). Statistical significance ($p \leq 0.05$) was determined with two-way analysis of variance (ANOVA) and a *post hoc* test namely, Bonferroni.

3.3 Results

3.3.1 Visualization of nuclei using fluorescent and light microscopy

A number of histological fixating, staining and detecting techniques, as listed in Table 1, were assessed in order to optimise the visualisation of genetic material, such as the nucleus and DNA in sugarcane cells, which ultimately would allow for the detection of micronuclei and their quantification.

Light microscopy

Sugarcane samples which were hydrolysed in HCl and used LR white for embedding, were stained using the Feulgen. These images showed mostly stained cell walls and nuclei (Figure 3.2). However, not all nuclei were stained and visualisation of nuclear material in all adjacent cells was not possible. Thus, this method would possibly allow visualization of micronuclei in single cells but quantification of micronuclei in a biological sample would have been impossible.

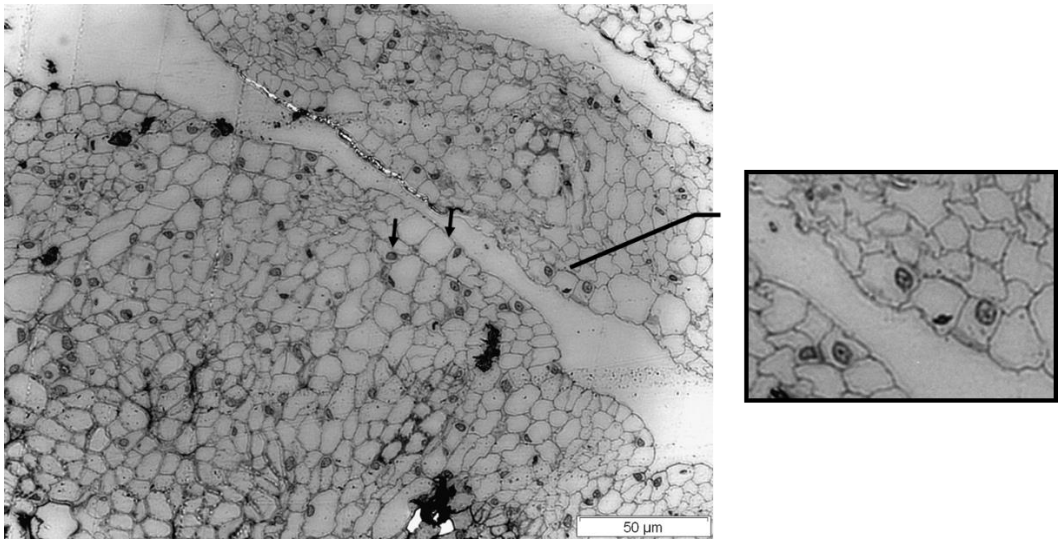

Figure 3.2: Light microscopy visualising images of sugarcane leaf discs after LR white embedding and staining according to the Feulgen method, including Schiff's reagent and toluidine blue, to identify chromosomal material and cell walls as indicated by the arrows. A 40x magnification was used and the size bar indicates 50µm.

When sugarcane tissue, callus and discs, were fixated overnight in formal saline, paraffin embedded, stained with H&E and sectioned with a microtome, clear cell outlines with the nucleus inside could be observed (Figure 3.3). Haematoxylin and eosin combination stain, stain nuclear material blue and the cytoplasm and extracellular material pink, respectively. However, it was sometimes difficult to distinguish between variation in the blue (violet) to pink stain, representing nuclei and other cellular components.

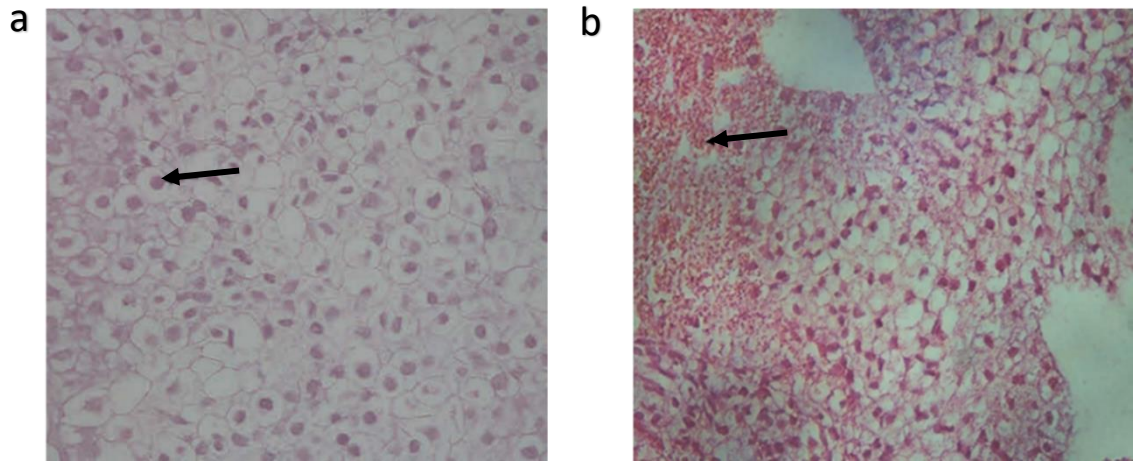

Figure 3.3: Light microscopy visualising images of sugarcane **a)** callus and **b)** leaf discs after paraffin embedding and H&E staining. Arrows indicate violet staining of nuclei (a) and pink staining of extracellular matrix (b). A 40x magnification was used and size bar indicates 50 µm in both images.

Fluorescent microscopy

Furthermore, sugarcane tissue samples were fixed in formal saline, stained with Hoechst and embedded in paraffin wax for easier sectioning with a microtome. However, after fixation, staining, and visualisation it was clear that not all nuclear material was stained. Furthermore, microtome sectioning resulted in overlapping cells, which made it difficult to identify nuclei (Figure 3.4). Thus, this method was not continued on sugarcane discs.

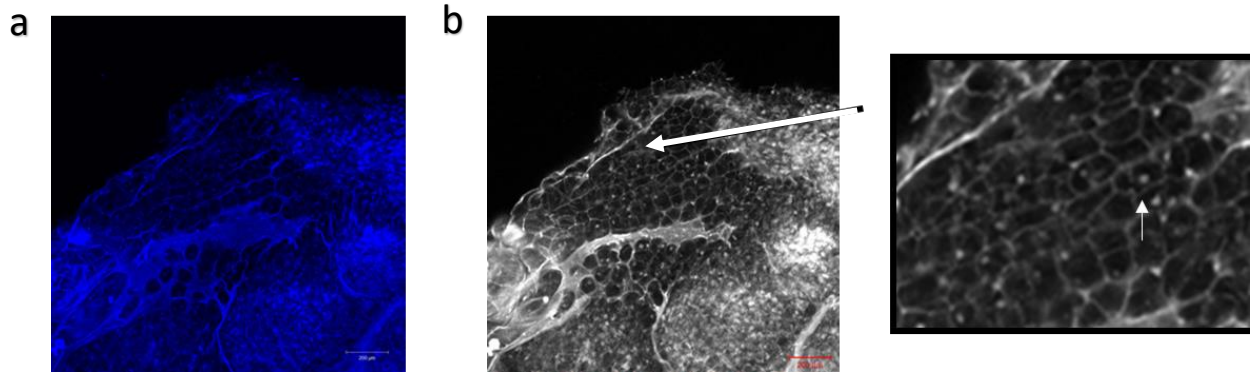

Figure 3.4: a) Sugarcane callus stained with Hoechst after fixation using a blue filter. **b)** Sugarcane callus stained with Hoechst after fixation without the blue filter to make identification easier. Image was taken at a 40x magnification and the size bar represents 200 µm. Arrow indicates stained nucleus.

Sugarcane callus and leaf disc tissue were fixated overnight in isopentane and freezing medium, sectioned with a cryostat and stained with propidium iodide (PI) and SYTO 9 (Figure 3.5). PI is a red- and SYTO 9 a green-fluorescent stain that can detect DNA content in cells. However, both PI and SYTO9 had difficulty penetrating living cells and very limited red and green DNA staining within the cells could be seen in the microscope images (Figure 3.5C). Also, the sectioning using the cryostat was less successful as thin sections resulted in the compromised integrity of the cellular components. However, when thicker sections were made, overlapping layers of cells being stained together making clear focused images difficult to obtain. Thus, micronuclei could not be visualised using these staining methods.

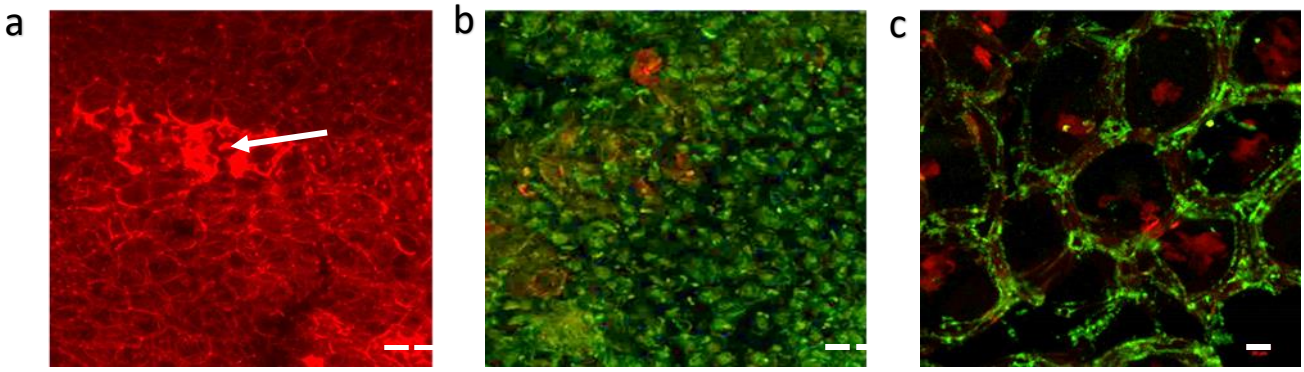

Figure 3.5: Fluorescent microscopy visualising images of sugarcane **a, b)** callus and **c)** leaf discs after PI and SYTO 9 staining using 40x **(a, b)** and 100x **(c)** magnification. Arrow indicate penetration of PI stain in a damage callus cell. Images (b) and (c) are stained with both dyes, and filters were changed to view each colour dye and then overlaid to create a cohesive image. Indicated size bars represents **a, b)** 200µm **c)** 20 µm.

Sugarcane callus cells, not leaf discs, were digested using pectinase and also stained with PI and SYTO 9 to detect DNA content (Figure 3.6). Stained cells in liquid suspension were pipetted onto microscope slides and visualised through fluorescent microscopy. However, the individual cells still tended to cluster together, which made imaging challenging (Figure 3.6c). It was unclear whether some of the SYTO 9 green fluorescent stained cell content were micronuclei since the nuclei material seem to be fragmented (Figure 3.6a). PI staining was also unable to penetrate the cell membrane of healthy cells, thus not staining the nuclear material inside these cells (Figure 3.6b).

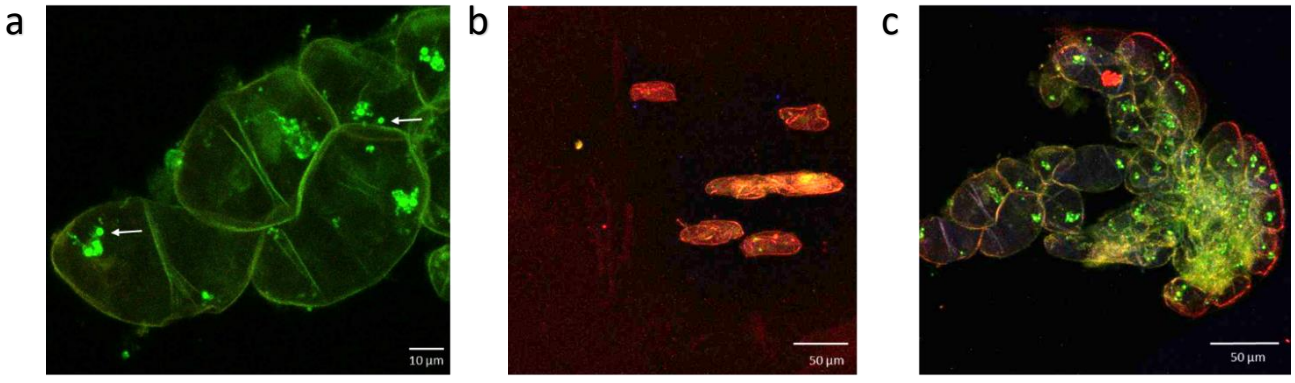

Figure 3.6: Fluorescent microscopy visualising images of irradiated sugarcane callus cells stained with **a)** SYTO 9, **b)** PI and **c)** SYTO 9 and PI using 40x magnification. In image (a) green staining of fragmented nuclear material are seen, some spots might be micronuclei indicated by the arrows; (b), the PI dye penetrated only into damage calli cells; in (c) clusters of stained cells are visible. Scale bars **a)** 10 µm **b)** 50 µm and **c)** 50 µm.

DAPI was used to stain callus and leaf disc samples in a process that comprised of fixation in formal saline. Using DAPI staining, cell outlines and nuclei could be clearly detected and micronuclei could be identified in the sugarcane cell tissue (Figure 3.7).

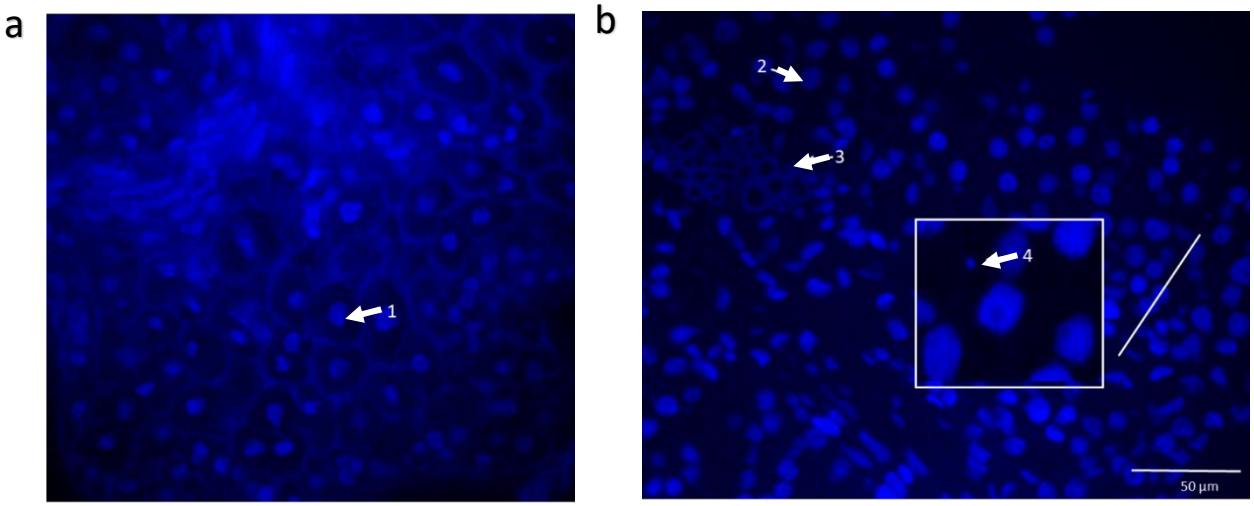

Figure 3.7: Fluorescent microscopy visualising images of irradiated sugarcane **a)** callus and **b)** leaf discs after DAPI staining using 40x magnification. Arrows indicate (1) nucleus in callus cell; (2) nucleolus in nucleus; (3) vascular tissue; (4) micronucleus. Scale bar indicates 50 µm for both images.

SEM and STEM

SEM and STEM images of the surface and internal structures of the leaf disc and callus cells were generated. In preparation, tissue was fixated, dehydrated, sectioned and mounted on aluminium stubs followed by gold coating which enhanced the conductivity necessary for electron flow. No image of micronuclei could be detected using either of these two methods (Figure 3.8). SEM could only visualise cell surface structures, while STEM was successful in visualising the cells and their components but could only detect chromatin and no micronuclei close to the nucleus. It is also only by chance, or by doing a large number of mounted sections, that ultramicrotome sectioning will cut at a level through the cell that will result in visualizing the cell's DNA content.

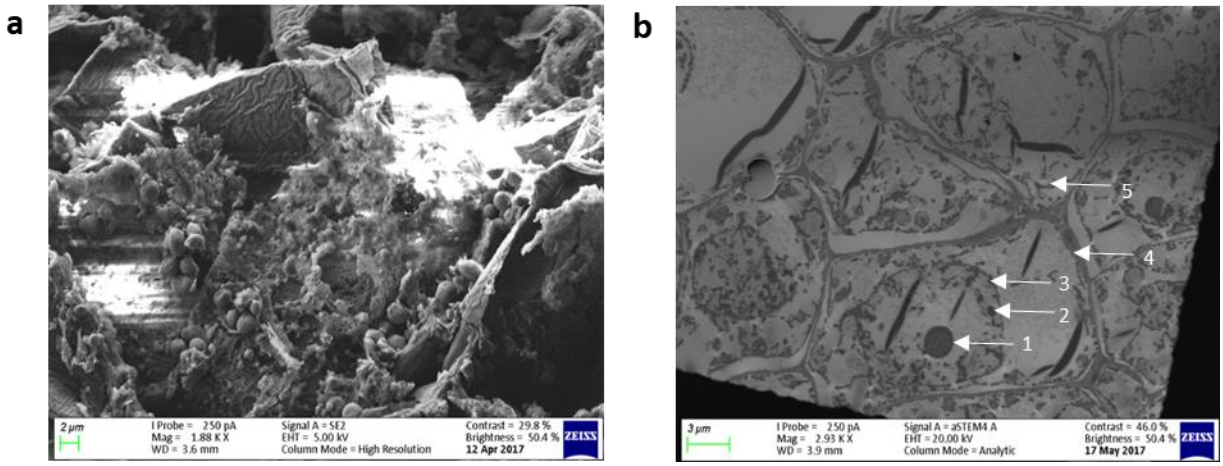

Figure 3.8: Structure of irradiated sugarcane cells image by means of **a)** scanning electron microscopy using sugarcane discs and **b)** scanning transmission electron microscopy using sugarcane callus. Size bar represent a size of 2 µm (a) and 3 µm (b), respectively. Arrows indicate (1) nucleolus, (2) chromatin, (3) nuclear envelope, (4) cell wall and (5) cell membrane.

3.3.2 Quantification of micronucleus formation in irradiated sugarcane cells

Using the DAPI staining method, the dose-rate effect of gamma radiation on micronucleus formation in sugarcane callus cells were investigated. The total frequency of gamma radiation-induced micronuclei varied depending on dose and post-radiation incubation times. The average frequency of micronuclei after gamma radiation varied from less than 20 micronuclei for 10 Gy, between 30-40 micronuclei for 20 Gy and between 20-30 micronuclei for 40 Gy, across all post-radiation time periods (Figure 3.9). Gamma radiation at 20 Gy induced a significantly higher number of micronuclei very early, 12 h, after radiation when compared to the non-radiated tissue samples. The dosages of 20 Gy and 40 Gy also induced high numbers of micronuclei 36 h after radiation.

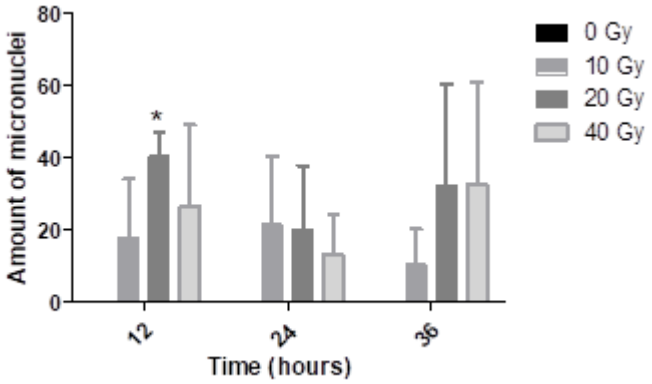

Figure 3.9: Frequency of micronuclei in sugarcane callus cells, 12, 24 and 36 hours after gamma irradiation. Significant difference ($p<0.05$) is indicated with (*). A 2-way ANOVA and a Bonferroni *post hoc* test was used to determine significant differences between samples.

3.4 Discussion

Micronuclei formation has been viewed as a viable genotoxic marker for radiation damage (Luzhna *et al*., 2013). Information regarding the induction and extent of DNA damage in plant chromosomes, linked to mutagenic treatment dosages, such as the formation of micronuclei, can be useful in mutation breeding programs. In this study we aimed to establish a method to detect micronuclei in sugarcane tissue and determine whether micronuclei formation is dependent on the radiation dose-rate.

Several different histology methods were investigated in an effort to optimise the detection of micronuclei in the sugarcane cells. A number of useful histological tools and techniques are available to detect damage to DNA and chromosomes. However, histological imaging in plants can be challenging and is complicated by auto-fluorescence and the impermeability of plant cell walls. It is possible to overcome these by the removal of autofluorescence through chemical treatments and enzymatic digestions of cell walls. Alternatively, dyes with emission spectra outside the auto-fluorescence wavelengths or filters blocking excitation in the 420 to 460 nm range can also be utilised (Soukup, 2014). In this study we analysed difference tissue preparation methods and dyes in their ability to detect micronuclei in sugarcane cells.

Tissue preparation for plant cell imaging involves possible fixation, hydrolysis and dehydration of cells and can influence the success of detecting cell structures. Fixation is used to freeze a sample in place in terms of its development. Thus, fixation maintains the integrity of a sample with minimal changes in cellular structures (Suvarna *et al*., 2012). It therefore stops all ongoing biochemical processes and make cells stable, which will in turn allow for the preparation of thin, stained tissue sections. Fixation can be done through heat, chemicals such as aldehydes, alcohols, oxidizing agents, or even freezing of tissue (Suvarna *et al*., 2012). Dehydration, specifically through coagulant fixatives, is most

commonly achieved using alcohols such as ethanol and methanol. Dehydration causes the removal of water from the cells due to the interaction between the alcohol and the water molecules present in the cells (Lawrence, 2008). Hydrolysis can be seen as a chemical method to induce the process of decomposition. In this process, water is used to cleave chemical bonds of certain structures found in cells through the use of acids or enzymes (Rogoff and Screve, 2011; Speight, 2017). For example, the acid hydrolysis process can remove purine bases thereby opening aldehyde groups which can then react with stains to dye nucleic acids.

Histological staining procedures allow for the visualization and highlighting of specific tissue structures and features by enhancing tissue contrast (Alturkistani *et al*., 2015). Different stains were tested for the visualization of nuclear material in the sugarcane cells. All dyes, except H&E, were used for fluorescent imaging. Firstly, toluidine blue, is a basic thiazine metachromatic dye, which stains components such as RNA and DNA blue due to its affinity for acidic structures (Sridharan and Shankar, 2012). The Feulgen staining technique which was used to try and quantify micronuclei, was initially used due to its popularity in microscopy for plants (Braselton *et al.,* 1996). In this staining technique Schiff's reagent was used in an attempt to stain cell walls, while toluidine blue dye was used in conjunction to dye nuclear material. These two dyes were used together so as to be able to identify nuclear material in individual cells (Figure 3.2) However, this technique, mostly used in the past in histology imaging of root tissue (Braselton *et al*., 1996; Fox, 1969), which differs greatly from sugarcane callus and leaf roll tissue, was unsuccessful. Only a limited number of nuclei present in the tissue sections were stained, and this technique was eliminated.

Using haematoxylin and eosin staining (H&E), nuclei could be identified and therefore possibly also micronuclei (Figure 3.3). H & E stain is a popular general-purpose stain where hematoxylin stains nucleic acids blue due to binding of the dye to acidic structures, while eosin stains multiple cell structures such as proteins, fibers, collagen and the cytosol a light pink colour due to the dye binding to basic structures (Fischer *et al*., 2008).

Propidium iodide (PI) has been used extensively in staining of cells, including plant cells (Arumuganathan and Earle, 1991). PI is a fluorescent agent that stains DNA and RNA, including the nucleus and DNA-containing organelles by intercalating between bases (Darzynkiewicz, 2011). In the past it has been especially used to evaluate cell viability since it has difficulty crossing the cell membranes of living cells (Boyd *et al.,* 2008; Jones *et al*., 2016; Musielak *et al*., 2015). Staining of the sugarcane cells with PI was unsuccessful as it stained the outer cell layers and cell surface but penetration to deeper tissue parts was not efficient, except when the cell membranes were clearly damaged (Figure 3.5). This was also seen in studies conducted by Truernit and Haseloff (2008) and

Jones *et al*. (2016) where PI could only enter and stain intracellular components, in *Arabidopsis* and rice respectively, when the membranes were damaged leading to permeability of this dye.

Also tested was SYTO 9, which is a green fluorescent cell-permeant nucleic acid staining dye (Stiefel *et al*., 2015). It can penetrate virtually all cell membranes including plant cells (Berney *et al*., 2007; Thomas and Sekhar, 2014), has a low intrinsic fluorescence when not bound to nucleic acids and a large fluorescence enhancement when bound to DNA/RNA (Stiefel *et al*., 2015). It acts, however, not as an exclusive nuclear stain, when compared to DNA-selective compounds such as DAPI, and can also show cytoplasmic staining. It has extensively been used to stain DNA content in bacteria (Stiefel *et al*., 2015) and in a few cases also plant cells such as banana and *Medicago truncatula* (Cam *et al*., 2012; Thomas and Sekhar, 2014). When sugarcane cells were stained with SYTO 9, green fluorescence was seen in both cell perimeter tissue and possibly the nucleus (Figure 3.3). However, the tissues stained with PI and SYTO9 were prepared by sectioning with the cryostat which resulted in multilayer cell sections making clear imaging difficult. In an attempt to reduce cell stacks for enhance focussing of cell images sugarcane callus were digested with pectinase and stained with PI and/or SYTO 9 (Figure 3.6). Pectinases are enzymes that can degrade the pectin found in the cell walls of higher plants resulting in protoplast formation (Gupta, 2016). Individual cells with their green fluorescing nuclei and possible micronuclei were seen in the microscope images but quantification of micronuclei formation in these single cell tissue samples would not have been possible, as the micronuclei were not all visible. Furthermore, the process of finding enough cells to count would have taken too much time. In addition, even after treatment with pectinase, cell clumps were still present making clear imaging of the cells problematic.

Lastly, sugarcane tissue was stained with DAPI. DAPI is a blue fluorescent stain which binds to especially adenine-thymine (A-T) regions of DNA and to a lesser extend with RNA (Chazotte, 2011; Kapuscinski, 1995). This compound can move through cell membranes and can stain both viable and fixed cells (Abcam.com, 2019; Gomes *et al*., 2013). It has been used in the past to stain double stranded DNA in plant such as petunia (Kamo and Griesbach, 1993) and tobacco (Ulrich and Ulrich, 1986). In this study it was used as an alternative to H&E staining in an effort to add more contrast to distinguish between different nuclear cell components (Figure 3.7). With DAPI staining of the sugarcane tissue we were able to clearly detect the plant cell nucleus as well as small nuclear fragments, that were identified as micronuclei (Figure 3.7). The micronuclei in these images are about a third of the size of the normal nucleus in the cells, also described as such by Luzhna *et al*., (2013).

When sugarcane cells were visualised with scanning electron microscopy (SEM), only images of the outside of the cells were seen with no cell content visible, thus making it impossible to view the inside

of the cells and therefore the nuclei and micronuclei. This was confirmed by Pathan *et al*., (2010) who visualised plant leaf surfaces in the goosefoot species (*Chenopodium*). The STEM imaging was more successful in visualizing the cell content, including the cell nucleus (Figure 3.8). However, visualising and especially quantifying the micronuclei with this technique seemed improbable since several different sections of the sugarcane samples had to be scanned and viewed to find one image showing nuclear material.

The micronuclei induced by a mutagen such as gamma radiation could derive from acentric fragments after chromosome breakage or from chromosomes lagging due to the dysfunction of cellular mitosis (Luzhna *et al*., 2013). Thus, the formation of micronuclei could be an indication of the radiation dosage damage to sugarcane samples. To assess this possible correlation, the quantification of micronuclei using the images gained from multiple tissue preparations and DAPI staining of the prepared sections were assessed (Figure 3.7). Different numbers of micronuclei were detected for tissue exposed to the different irradiation dosages. No clear correlation was seen between increase in radiation dosage and micronucleus formation in the sugarcane cells. However, all tissue exposure to radiation showed formation of some micronuclei, with the higher dosages showing higher micronuclei formation, however the higher formation of micronuclei was not significantly different to the control samples (Figure 3.9), even though non-irradiated tissue showed no formation of micronuclei. In addition, the formation of micronuclei tended to increase and decrease over time as cell division progressed. It is known that micronuclei are formed due to damage to the genome and are capable of DNA repair as well as transcription and replication (Hintzsche *et al*., 2017). Micronuclei can also be degraded through several processes including degradation through enzymatic actions as well as apoptosis and autophagy (Hintzsche *et al*., 2017; Kisurina-Evgenieva *et al*., 2016). Thus, even though micronucleus formation could not be linked to specific irradiation dose rate in this study, it can still be use as a genotoxicity marker for overall genome damage in sugarcane cells exposed to radiation.

3.5 References

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Chapter 4: PHYSIOLOGICAL AND BIOCHEMICAL ANALYSIS OF MUTANT SUGARCANE LINES, GENERATED THROUGH THE APPLICATION OF EMS, TO DETERMINE DROUGHT TOLERANCE

4.1 Introduction

Water deficit is one of the most detrimental environmental stresses affecting sustainable crop production across the globe. In South Africa, sustainable agriculture is an important contributor towards the country's economy and especially affects small-scale farmers who rely on crop production for their own food security and the wellbeing of their families and communities (Pillay, 2016). Sugarcane, as with many other plant species, shows detrimental effects when confronted by a decrease in available water and water shortages can result in production losses of up to 60% (Basnayake *et al*., 2012; Gentile *et al*., 2015).

Optimal sugarcane production is dependent on receiving relatively high amounts of water throughout its development; in South Africa an estimated annual minimum of 1500 mm is needed for growth and sugar production (DAFF, 2014). In South Africa, 85% of land for sugarcane crop production is rain fed and therefore relies heavily on regular rainfall (Carr and Knox, 2011). Thus, it is necessary to create sugarcane genotypes which can sustain their yield production through various biochemical and physiological drought-response mechanisms, when faced with a lack of rainfall and available irrigation.

Mutation induction in plants is seen as a relatively easy technique to induce genomic changes that can result in phenotypic alterations such as increased resistance to pest/pathogens and increased tolerance to environmental stressors. Mostly mutation breeding has been applied in economic important crops, as well as legumes and flowers. Over 200 species of mutated plants have been registered and are available for commercial use (FAO, 2018).

Mutation breeding, through chemical mutagenesis, has been used in the past to introduce many beneficial genetic mutations in plant species. To date, there are 375 officially registered plant species that have been mutated using different forms of chemical mutagens, these include for example plant species such as tobacco, wheat, flaxseeds, lentils, barley and buckwheat (FAO/IAEA-MVD, 2018 [https://mvd.iaea.org]). However, of these only 9 registered plant species have an increased drought tolerant phenotype, which include millet, sorghum, barley, maize, *Amaranthus* sp. and Sudan grass (*Sorghum sudanense*). Mutagenesis in these species have been achieved using mutagens such as dimethyl sulfate (DMS), ethyleneoxide, 4-dimethylaminoazobenzene (DAB), dioxane, N-nitroso-N-methylurea (NMU) and nitroso-ethyl urea (NEU) (FAO/IAEA-MVD, 2018). However, as of yet, no

mutants have been registered on the FAO/IAEA database that show increased drought tolerance through chemical mutagenesis using ethyl methanesulfonate (EMS). Currently, the registered EMS mutants have positively gained traits such as early flowering, colour change in petals, resistance to disease, higher yield, better tillering, and improved seed/flower colour in a variety of species. Even though no EMS generated crop varieties are registered for abiotic tolerance, numerous studies have been conducted to generate such plants. Several studies have been successful in generating drought tolerant mutant plant species such as banana (Bidabadi *et al.,* 2012), *Arabidopsis thaliana* (Chen *et al*., 2005) and tomato (Sala *et al*., 1990).

EMS is an ethylating agent that has been shown to cause DNA point mutations in a large number of organisms, including plants (Weil and Monde, 2009). These small point mutations can occur at high frequency, evenly spread out, in the plant's genome (Kriz and Larkins, 2009; Lodish *et al.*, 2000; Rhease and Boetker, 1973; Sega, 1984). Essentially, using EMS results in the mispairing of guanine residues with thymine residues instead of cytosine residues, which leads to G/C to A/T transitions (Talebi *et al*., 2012). EMS mutagen treatment in sugarcane has been used to develop varieties that show tolerance to heavy metals, changes in tiller and internodes numbers, millable cane and stalk colour and tolerance to *Fusarium sacchari* (Mahlanza *et al*., 2013; Mahmud *et al*., 2016; Purnamaningsih and Hutami, 2016)

For this study, putatively drought tolerant sugarcane plants (*Saccharum* species hybrid cv. N19) were created by exposing sugarcane callus to EMS and *in vitro* osmotic selection on polyethylene glycol (PEG) (Masoabi *et al*., 2017). PEG is a water soluble, non-ionic osmotic stress agent that can mimic the effects of drought by decreasing the water potential in *in vitro* growth media (Kocheva and Georgiev, 2003; Tsago *et al*., 2013). PEG is an ideal osmoticum since it is not absorbed by the plant's cells (Rao and Jabeen, 2013). *In vitro* pre-screening for abiotic tolerance has the advantage that it can reduce the number of potential mutants with desired traits prior to confirmation testing under field or glasshouse conditions. It also provides a means for easy cloning of selected mutants through micro-propagation. However, *in vitro* cultured cells often express different sets of genes when compared to whole plants growing in natural field environments. It is therefore important that any mutant plant, identified through *in vitro* selection systems, must ultimately be tested for the trait in greenhouse or under field conditions. This study aimed to confirm the tolerance to water stress in EMS generated osmotic tolerant mutant sugarcane plants in greenhouse pot trials.

4.2 Material and methods

4.2.1 Plant material

Mutant plant lines from *Saccharum* species hybrid cv. N19 were created by Masoabi *et al.* (2017). Briefly, N19 callus generated *in vitro*, was treated with 20 mM EMS and subjected to *in vitro* selection on 20% PEG. Treated calli were kept on growth media containing PEG in the dark for 8 weeks, allowed 2 weeks for recovery in the light on growth media without PEG, followed by an additional 8 weeks of PEG selection also under light growth room conditions (16 h/8 h light/dark regime, at 25°C; cool fluorescent light intensity). For this study, surviving plantlets were multiplied *in vitro* on semi-solid MS media with vitamins (Murashige and Skoog, 1962) containing 20 g/l sucrose, 0.1 mg/l BAP (6-benzylaminopurine), 0.5 g /l casein and 2.2 g/l gelrite at pH5.8. Plantlets, about 5 cm tall, were hardened off in the glasshouse at 26°C under natural light conditions by planting them in a mix of potting soil:sand:vermiculite (2:1:1 ratio). These plantlets were then covered with plastic bags to prevent wilting until the plants acclimatised in the drier glasshouse environment for atleast 2 weeks.

4.2.2 Phenotypic analysis and determination of carbohydrate content

For the assessment of normal growth and development, *ex vitro* acclimatised wild type (WT) N19 and mutant plants, lines M1, M2 and M3, were transferred to big 43 cm pots containing a mix of potting soil and sand, (2:1 ratio) and placed in a growth tunnel under natural sun light and temperature, on Welgevallen Experiment farm, Stellenbosch University, Stellenbosch. Pots were equipped with a dripper irrigation system containing nutrients (Multifeed TM, South Africa) and received water on a daily basis. Plants were allowed to mature for 8 months.

After 8 months, plant growth was assessed by measuring internode length and diameter, plant shoot length, and leaf length and width. Measurements were conducted in triplicate (three stalks) from three plants of each line. Plant shoot height was measured from soil level to the top visible dewlap leaf (TVD), the TVD leaf and internode 9 were used for all leaf and stalk measurements, respectively.

In addition, the soluble carbohydrate content, namely sucrose, glucose and fructose, were determined in the stalk material of the WT and the mutant plants lines. For this, sugarcane internodes 3 and 9 were harvested, frozen and ground into a fine powder and 25 mg was used for sugar extraction. Sugars were extracted with the addition of 250 µl of 80% (v/v) ethanol, incubation at 95°C for 30 min with shaking, this step was repeated twice. After each incubation, samples were centrifuged at 10 000 g

for 10 min and the supernatant collected. For the last extraction the ethanol concentration was lowered to 50% (v/v). Supernatants from the three extractions for each sample were combined and stored at -20°C until measurements were performed. Carbohydrate content was measured using the Saccharose/D-glucose/D-fructose kit from R-Biopharm (Boehringer Mannheim, Darmstadt, Germany) following the instructions of the manufacturer using the Versa Max plate reader (absorbance read at 340 nm) and levels were determined as mmol/g fresh weight (FW).

4.2.3 Drought pot trial

Hardened off plantlets were transferred to 20 cm pots containing 2 kg of a mix consisting of peat:sand:vermiculite (2:1:1 ratio). These plants were placed in a glasshouse at 26°C under natural sun light and irrigated regularly. A week after planting, plants received 100 ml fertiliser consisting of 2.5 g/L calcium nitrate and 3 g/L Hygrotech nutrient salts (Hygrotech, South Africa), this treatment was repeated every 2 weeks. Sugarcane plants were allowed to grow for 3 months, until the first internode was visible above the soil level. At this stage the drought trail was initiated (day 0) and plants received no water for 21 or 28 days (depending on senescence) followed by re-watering to monitor recovery.

Leaf material (leaves 1 to 4) was harvested on days 0, 7, 14 and 21 and 28 (if possible) days without water (ww). Leaf 1 was the TVD leaf, and the subsequent leaves underneath it were numbered as leaves 2 to 4. Immediately after harvesting (leaves were cut with sterilised scissors), the leaves were flash frozen in liquid nitrogen. Samples were then stored at -80°C until processing.

4.2.4 Relative water-, soil moisture content and root mass

A ProCheck (Decagon Devices, USA) soil moisture probe was used to monitor the soil moisture content before withholding water and during the soil-drying period. The probe was inserted into the soil, at a depth of 10 cm, taking 3 measurements around the plant stem, to determine the average moisture content of each pot included in the trial.

Relative water content (RWC) of leaf samples of WT N19 and mutant lines exposed to water deficit were determined following the method describe by Smart and Bingham (1974). Leaf material from the TVD, consisting of leaf discs with a diameter of 1 cm, was harvested on day 0, 7, 14 and 21 pw. Leaf fresh, full turgor and dry weights were determined. Fresh weight was recorded immediately after harvesting of the leaf disc and full turgor weight was recorded after immersion of the leaf disc in

distilled water for 24 hours. Leaf samples were dried for 2 days at 60°C to determine the dry weight of the leaf tissue. The relative moisture content was calculated using the following equation based on the data collected from 3 leaf discs from 3 plants for each line:

$$RWC = \frac{fresh\ weight - dry\ weight}{Turgor\ weight - dry\ weight}\ x\ 100$$

For root mass, the fresh weight of the roots was measured on a scale and the weights were recorded. The roots were then placed in an oven at 60°C to dry out for 24 hours. The weight was then recorded and the fresh weight and dry weight were subtracted from each other so as to determine the weight of the roots.

4.2.5 Stomatal conductance, chlorophyll fluorescence (Fv/Fm) and chlorophyll content

A leaf porometer, model SC-1, (Decagon Devices Inc., USA) was used to determine the average leaf stomatal conductance as previously describe by Zarco-Tejeda *et al*. (2000). Measurements were taken in triplicate at three positions, top, middle and bottom, on the leaf surface of the top visible dewlap leaf (TVD) of three different plants. Stomatal conductance was expressed as mmol $m^{-2}s^{-1}$ at each time point according to the manufacturer's instructions.

The rate of photosynthesis, as a chlorophyll fluorescence ration (Fv/Fm), was measured according to Zhao *et al*. (2013) using a hand-held Chlorophyll Fluorometer (OS30p+; Opti-Science Inc., USA). Dark adaptation clips were applied to leaves at the mid-point of the TVD leaf for 20 min prior to reading. All measurements were taken in triplicate from three different plants. Chlorophyll fluorescence was expressed as fluorescence ratio, Fv/Fm. Multiple readings, both stomatal conductance and fluorescence ratio, were recorded at the onset of the water stress and repeated at 3-day intervals till 28 days pw.

Chlorophyll extraction was done according to the method of Lichtenthaler (1987) using 80% acetone. Briefly, sugarcane leaf sections were harvested, frozen in liquid nitrogen and ground to a fine powder. Tissue samples, 25 mg, were then placed into Eppendorf tubes and 2 ml 80% acetone was added and incubated overnight, or until the acetone solution became saturated, on a shaking incubator at 25°C. Tissue extractions were then centrifuged at maximum speed for 2 min, after which the supernatant was collected and stored at room temperature. The process was repeated until the solvent was clear, showing no green pigmentation. The supernatant of the repeated extractions was combined and the chlorophyll concentrations were spectrophotometrically determined at 645 nm and 663 nm using a

Versa Max plate reader (Molecular Devices, USA). The following equation was used to calculate total chlorophyll content according to the formula described by Arnon (1949):

$$\text{Total chlorophyll (mg/ml)} = \big(20.2(A645) + 8.02(A663)\big)\, x \frac{Volume\ (ml)}{1000\ x\ weight}$$

All measurements were taken at the beginning of the water stress treatment (day 0) and then at days 7, 14, 21, 28 pw of the WT and mutant plant lines. All measurements were carried out with three biological repeats and conducted in triplicate.

4.2.6 Electrical conductivity and malondialdehyde (MDA) content

The electrical conductivity (EC) of the sugarcane leaf material from the WT and mutant lines exposed to water deficit conditions were determined using the EC/TDS Tester from Adwa (Hungary). The probe was calibrated using the Kemical Conductivity Standard. Briefly, leaf samples (2 cm in length) were harvested, placed in 2 ml deionised water (EC1) in a 12 well assay plate and incubated for 1 hour after which the electrical conductivity was recorded (EC2). Leaf samples were then removed and flash frozen in liquid nitrogen, in a cold container, so that all material could be carried over effectively. Crushed leaf samples were placed back in deionised water and incubated on a shaker for 30 min, after which the EC was once again recorded for each sample (EC3). Measurements were taken in triplicate from three biological repeats. EC content was calculated using the following equation and measured in siemens per min (S/m) (Kirkham, 2014):

$$\text{Relative electrolyte leakage} = EC2 - EC1\ /\ EC3\ x\ 100$$

MDA content was determined based on the method describe by Patade *et al*. (2011) as well as Heath and Packer (1968). Leaves (50 mg) of well-watered (day 0) and droughted plants, both WT and mutant lines, were harvested 0, 7, 14, 21- and 28-days pw. Leaf samples were snap-frozen in liquid N_2, ground to a fine powder and homogenised in 2 ml of 6% trichloroacetic acid (v/v; TCA). Samples were centrifuged at 4°C at 13000 g for 15 min and the supernatant collected. For MDA measurements, 200 µl of supernatant was mixed with 400 µl of 20% TCA and 0.05% triboric acid (v/v; TBA). Samples were vortexed, boiled at 90°C for 20 min, chilled on ice for 10 min and centrifuged at 13000 g for 15 min. Absorbance was measured at 532 nm and 600 nm and the concentration of MDA calculated using the

extinction coefficient of 155 $mM^{-1\ cm-1}$. All enzyme activity was measured on the Versa Max plate reader (Molecular Devices, USA). MDA content was calculated according to the formula:

nmol MDA/ mg FW = $((A_{532}-A_{600}) *D*x*1000)/Ɛ*ƃ*\bar{y})$

Where

- $A_{532}-A_{600}$ = The true absorbance corrected for non-specific absorbance
- D= Dilution factor of extract to reaction buffer
- Ɛ = Extinction coefficient 155 mM^{-1} cm^{-1}
- *X* = amount of TCA used in ml
- $\bar{y}$ = mg FW of tissue used
- ƃ = Distance travelled (0.63 cm)

4.2.7 Measurement of antioxidant levels

The Catalase Assay Kit from Sigma Aldrich (USA) was used to determine levels of catalase activity in the WT and mutant lines, following the manufacturer guidelines. Samples were prepared by homogenising 50 mg leaf sample in 0.1 M potassium phosphate buffer (pH 7) containing 1% (w/v) PVP (polyvinylpyrrolidone) and 0.1 mM EDTA (ethylenediaminetetraacetic acid). Samples were vortexed briefly and centrifuged at maximum speed for 5 min. The amount of remaining H_2O_2 (hydrogen peroxide) was determined by means of the formation of the quinoneimine dye that absorbs at 520 nm. Catalase activity was expressed as µmol/min/g FW. A standard curve was prepared by measuring the absorbance of the red quinoneimine dye versus a series of H_2O_2 concentrations ranging from 0 to 0.75 mM (Supplementary data Figure 6.1a). A stock solution of 10 mM hydrogen peroxide was used to prepare the dilution series for the linear curve.

The levels of superoxide dismutase (SOD) in the plant samples, WT and mutant lines exposed to water stress, were determined using the SOD Assay kit (Sigma Aldrich, USA) following the manufacturer's instructions. Plant samples were prepared in a similar way as for the catalase measurements. SOD was assayed through the measurement of water-soluble tetrazolium salt (WST) that produces a formazan dye measured at an absorbance of 440 nm. SOD activity was expressed as a percentage inhibition rate. A standard curve was setup by creating a dilution series of SOD (Sigma, USA) from 0.05 mM to 200 mM. The linear curve is presented in the Supplementary data (Figure 6.1b).

4.2.8 Histochemical detection of reactive oxygen species (ROS)

Determination of H_2O_2 was done by 3,3'diaminobenzidine (DAB) staining according to the method of Daudi and O'Brien (2012) and superoxide radicals (O_2^-) by nitroblue tetrazolium (NBT) staining following the method of Ramel *et al*. (2009). Leaf segments were collected from both WT N19 and mutant lines under both stressed and unstressed conditions during the course of the water deficit period. Briefly, the DAB staining solution was freshly made and consisted of 50 mg DAB dissolved in 45 ml H_2O, adjusted to pH3 by the addition of 0.05% (v/v) Tween 20 and 10 mM Na_2HPO_4. NBT staining solution was also made fresh on the day of use and consisted of 3 mg NBT dissolved in 100 ml of a 10 mM phosphate buffer, pH7 and 1 M NaN_3. The leaf samples were immersed in the staining solutions, placed under vacuum and left overnight on a shaker at room temperature, to ensure infiltration. Stained leaf segments were de-stained in a solution of ethanol:glycerol:acetic acid, ratio 3:1:1 (v/v), at 95°C until all chlorophyll had been removed, and stored in ethanol:glycerol, ratio 3:1 (v/v), until photographed.

4.2.9 Measurement of proline content

Proline content was determined based on the method describe by Bates *et al*. (1973). Leaf samples were ground in liquid N_2 and 50 mg extracted in 5 µl/mg FW (fresh weight) of 3% sulfosalicylic acid (v/v; SAS). Extracted samples were stored on ice and 500 µl SAS, glacial acetic acid and acidic ninhydrin (1:2:2 ratio) was added to 100 µl of extract. Acidic ninhydrin consisted of 1.25 g ninhydrin (Sigma Aldrich, USA) dissolved in 50 ml glacial acetic acid:orthophosphoric acid (6 M) (30:20 ml), slowly mixed together at 50°C. Samples were kept on ice, vortexed and centrifuged for 5 min at maximum speed in a desktop centrifuge. Sample supernatants were collected and heated to 95°C for 60 min, cooled to room temperature and the absorbance measured at 520 nm on the Versa Max plate reader. Proline activity was expressed as nmol/mg FW.

A standard curve was established by creating a dilution series of L-Proline ranging from 5 mM to 100 mM (Supplementary data, Figure 6.1c). Proline content was calculated using the following equation:

$$\frac{Absorbance\ of\ extract - absorbance\ of\ blank}{slope\ of\ standard\ curve} \ x\ \frac{Volume\ of\ extract}{Volume\ of\ aliquot} x\ \frac{1}{FW\ (mg)}$$

4.2.10 Description of statistics

All measurements were made with three biological repeats (N=3) with additional technical repeats done in triplicate (n=9). Mean values were presented with their standard error (SE). All graphs and the subsequent statistical analysis were completed using GraphPad Prism version 6.01 for Windows (GraphPad Software, La Jolla, USA). Statistical validation and significance ($p \leq 0.05$) were assessed with one-way or two-way analysis of variance (ANOVA) followed by the Bonferroni *post hoc* test (Bonferroni, 1936).

4.3 Results

4.3.1 Phenotypic analysis of plants under normal growth conditions

Wild type (WT) N19 plants and mutant sugarcane plants, lines M1, M2 and M3, were selected after the application of an *in vitro* osmotic stress regime (Masoabi *et al*., 2017). Three plants of each line were allowed to mature for 8 months in unstressed environmental conditions, after which they were subjected to phenotypic analysis. Unfortunately, due to irrigation problems at the experimental farm, lines M1 and M2 were lost and further experimentation were not possible. Plants from the WT control and M3 mutant line were assessed in regards to total shoot height, leaf length and width, stalk diameter and internode length (Table 4.1). M3 plants had a statistically significant increase in their total shoot height and total leaf length when compared to the WT, but no difference was seen for leaf width, stalk diameter and internode 9 length between the WT and mutant plants.

Table 4.1. Phenotypic parameters of mutant and WT plants allowed to mature for eight months under normal growth conditions. Statistical significance (*) was determined using a 2-way-ANOVA and a Bonferroni *post hoc* test, when mutant lines were compared to the WT plants ($p \leq 0.05$). Data represents the average values of 3 biological repeats.

	PHYSICAL PARAMETERS (CM)				
LINE	**Total shoot height (cm)**	**Leaf (TVD)(cm)**		**Internode 9 (cm)**	
		Length	**Width**	**Diameter**	**Length**
WT	132.10 ± 11.21	119.30 ± 1.61	7.00 ± 0.50	9.30 ± 0.25	9.80 ± 0.29
M3	190.40 ± 14.27 *	169.70 ± 18.82 *	5.70 ± 0.25	8.70 ± 0.30	11.60 ± 1.00

In addition, the carbohydrate content of the mutant plants was assessed and compare to levels found in the N19 WT plants. Sugar levels were determined from tissue harvested from internode 3 (immature) and internode 9 (mature). There were no significant differences in the sucrose, glucose or fructose content in immature cane from the mutant plants (M3) compared to the N19 WT plants (Figure 4.1). This was in contrast to the significantly higher levels of sucrose and glucose in M3 mature cane when compared to the WT.

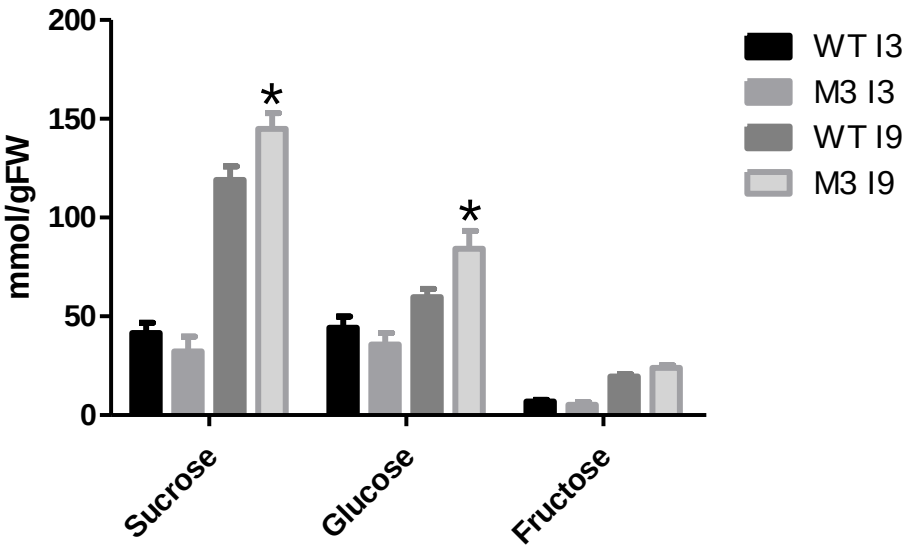

Figure 4.1: Soluble carbohydrate levels in eight-month-old WT N19 and mutant line M3 plants. Indicated are sucrose, glucose and fructose levels in immature (internode 3; I3) and mature (internode 9; I9) stem tissue. Significant differences ($p \leq 0.05$) between wild type (WT) and mutant lines were indicated by the (*) symbol and were determined using a 2-way ANOVA test and a Bonferroni *post hoc* test.

4.3.2 Phenotypic analysis of plants under water stress conditions

Plants, WT N19 and mutant lines M1, M2 and M3, were grown in the greenhouse for 3 months (Figure 4.2; day 0 pw panel). After 3 months the irrigation was switched off and the plants did not receive any more water for a period of 28 days (Figure 4.2). After 21 days of water stress the WT plants were the worst affected by the water stress, being completely dehydrated with all leaves being dry, brittle and yellow (Figure 4.2; day 21 and 28 pw panels). Plants of the mutant line M1 also showed signs of senescence with leaves displaying signs of bleaching and drying of mature leaf tips, but not to the

extent observed in the WT plants (Figure 4.2; day 21 pw panel). However, mutant lines M2 and M3 at 21 days pw, exhibited stay-green phenotypes that in the case of M2 even extended to 28 days pw. On average M1, M2 and M3 survived 5, 12, 10 days longer than the WT plants, respectively. After 28 days pw (M2) or earlier, if plants had only dried leaves left, plants were re-watered and received fertiliser. All WT, M1 and M3 plants were unable to recover from their dehydrated state while M2 was able to recover and regain its green colour and continue its growth process (Figure 4.2; recovery panel). The M2 phenotype after re-watering showed either resprouting or the old leaves showed regeneration of the young inner meristematic leaf roll tissue.

Root mass in general increased in both the WT and mutant plants during the course of the water stress period. Root mass between individual plants within a plant line varied considerably as indicated by the large standard error (SE) bars (Figure 4.3). No significant difference was seen between the root mass of the WT and mutant lines after the onset of drought conditions.

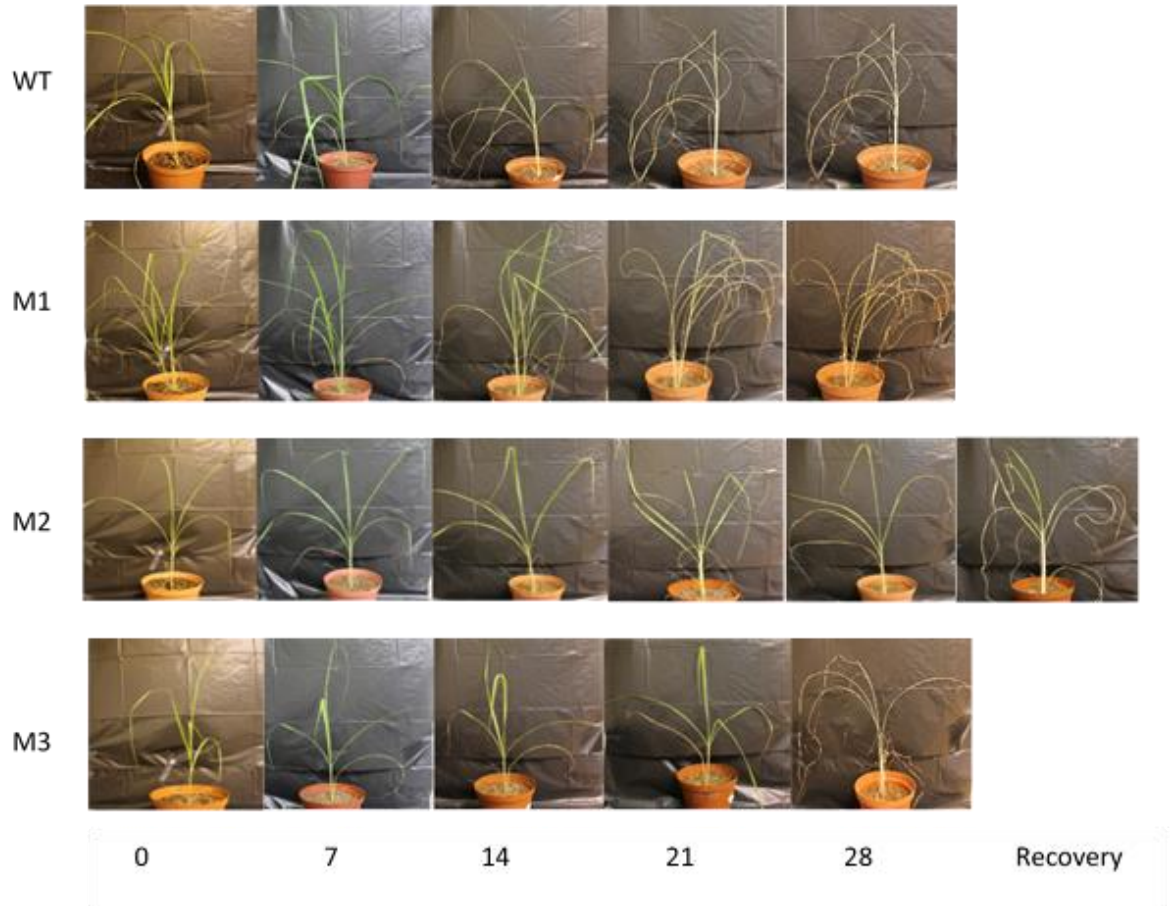

Figure 4.2: N19 wild type (WT) and mutant sugarcane plants (M1, M2 and M3) subjected to water stress. Panels indicated 3-months old plants before water stress (day 0), after 7, 14, 21 or 28 days without water and then 14 days after re-watering (Recovery panel). An absent photo panel indicates that the plants did not survive.

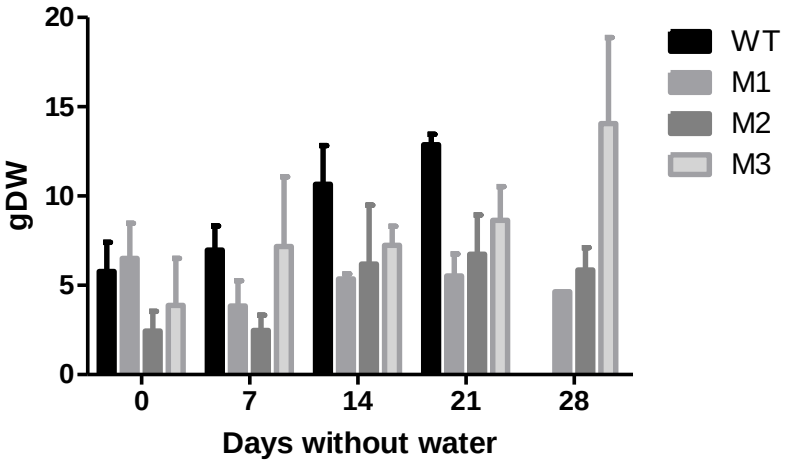

Figure 4.3: Root mass (dry weight), of WT and mutant lines over a period of 28 days of drought. Significant differences ($p \leq 0.05$) between WT and mutant lines were determined using a 2-way ANOVA, but no significant difference was seen between samples.

4.3.3 RWC and soil moisture content as a measurement of plant water status

The relative water contents (RWC) of the mutant and WT plants were measured at six time points namely day 0, 7, 10, 14, 21 and 28 pw, using material collected from the TVD (Figure 4.4). Overall, after 21 days of water stress, the WT plants lost an estimated 40% of their water content, while the RWC in mutant plants stayed almost constant (M1 line) or decreased by as little as 8% in the M2 and M3 mutant lines over the same drought period.

In addition, the soil moisture content of all the pots (n=25 / line) included in the trial were monitored. During the drought regime, the soil moisture content in all the pots decreased at more or less similar rates from ± 0.25 m^3/m^3 in the fully saturated soil at day 0 (beginning of trial) to less than 0.01 m^3/m^3 in the dry pots at day 21 pw (Figure 4.4b), around a 96% drop in moisture content. The soil moisture for pots containing M3 plants compared to the WT plants were significantly different on day 10 pw.

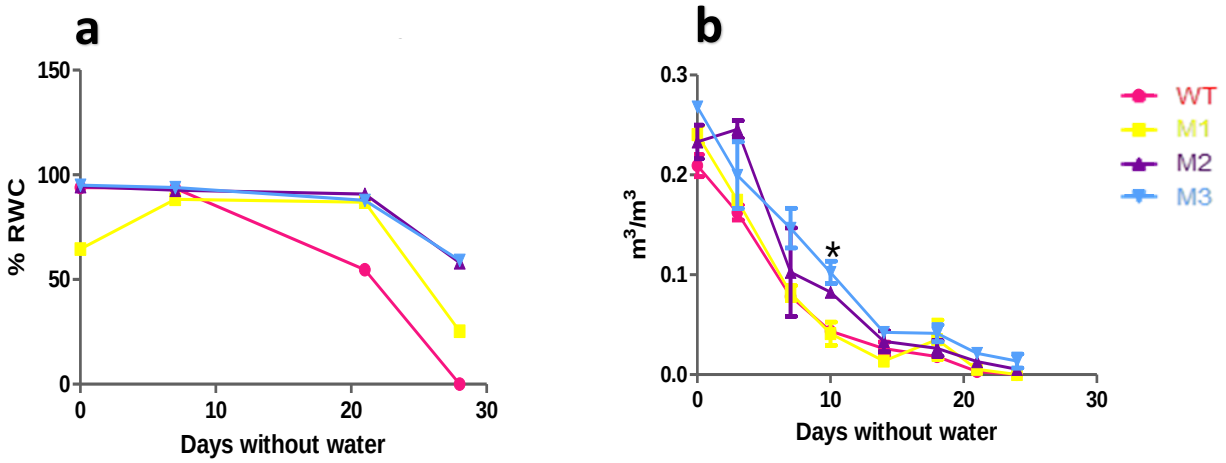

Figure 4.4: The influence of prolonged drought on the **a)** RWC and **b)** soil moisture content measured in the pots of the WT and mutant sugarcane plants (M1, M2, M3) over a 28 day stress period. RWC data represent the mean ± SE of 3 biological replicated (n = 3). Statistically significance, compared to the WT, at $p \leq 0.05$ is indicated with an (*) and was determined using a 2-way ANOVA.

4.3.4 Effects of drought on the photosynthetic machinery of the plants

Stomatal conductance and chlorophyll fluorescence were measured in WT N19 and mutant plants (M1, M2 and M3) as an indicator of the efficiency of photosynthetic performance. Stomatal conductance decreased over the course of the drought trial in all plants (Figure 4.5a). Only mutant line M3 displayed significant higher stomatal conductance when compared to WT plants on day 18, 21 and 24 pw. Day 24 pw was the last day readings were possible in some of the plants due to leaves turning dry and brittle.

Mutant lines M2 and M3 maintained higher levels of chlorophyll fluorescence, as detected in PSII (Photo system II) as a fluorescent ratio (Fv/Fm), from as early as day 14 pw when compared to the WT plants. Fluorescence levels were significantly higher in these mutant lines, when compared to the levels in the WT plants, on day 21 and 24 (Figure 4.5b). Furthermore, a drastic decrease in the fluorescence ratio as early as day 10 pw were observed in the WT and M1 plants, with high levels of variance seen between plants after this time point, as indicated by the large standard errors from the mean values. In contrast, the florescence ratio levels in mutant lines M2 and M3 stayed relatively constant throughout the drought regime (Figure 4.5b).

The chlorophyll content in WT and mutant plants prior to and under drought conditions were measured (Figure 4.5c). When quantitatively assessed, the total chlorophyll content was similar in all

plants, both WT and mutant plants, prior to the commencement of the stress period (day 0 pw) and stayed at constant levels during the early stages of the water stress (7 days pw). Towards the end of the stress period, when the plants were under severe water stress, the chlorophyll content in all the plants started to decrease (day 21 pw). However, mutant lines M2 and M3 seemingly maintained higher levels of chlorophyll, up till day 28 pw, with M3 plants showing significantly higher levels of chlorophyll in comparison to the WT plants.

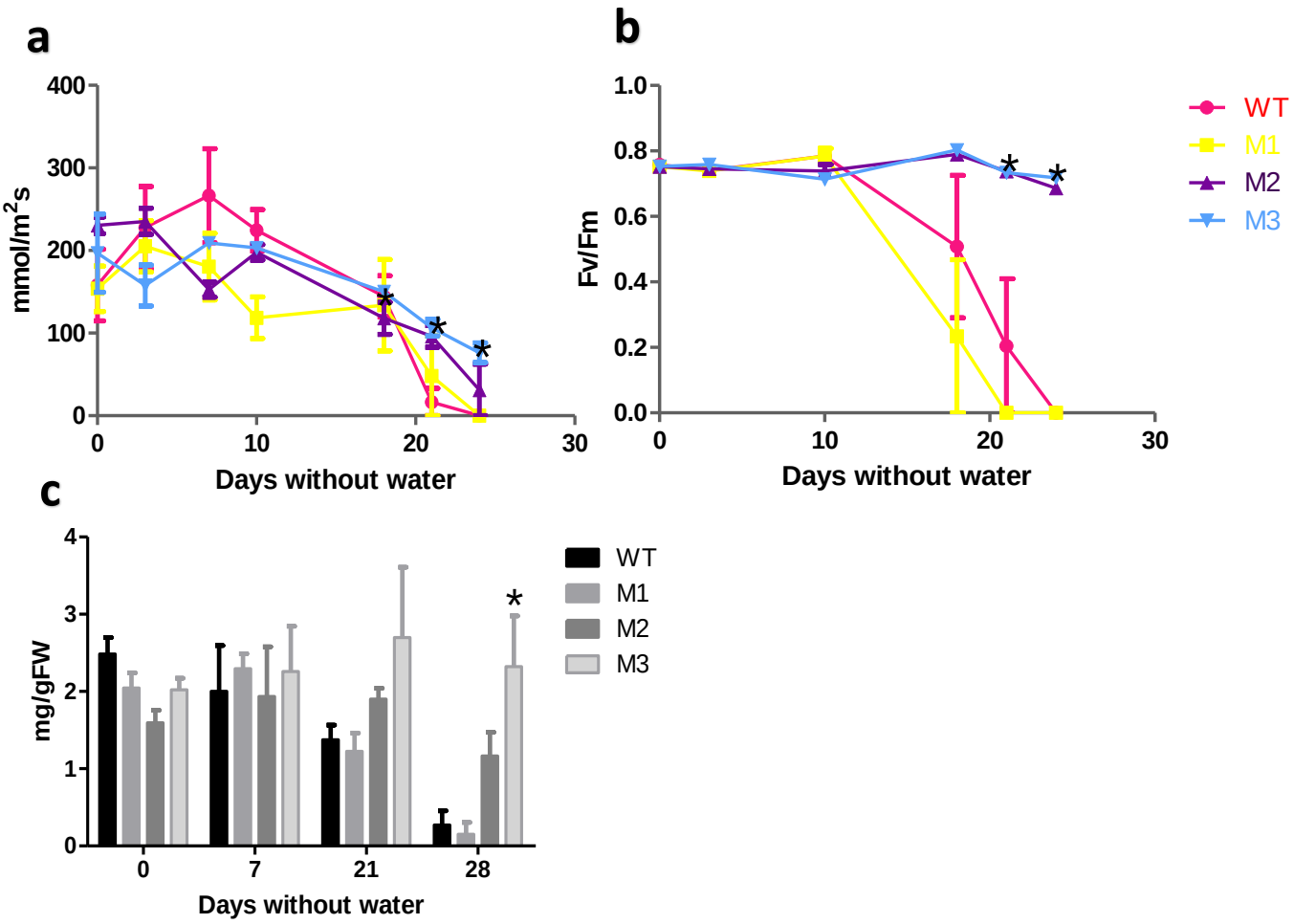

Figure 4.5: Influence of extended drought on **a)** stomatal conductance, **b)** chlorophyll fluorescence (Fv/Fm) and **c)** chlorophyll content in mutant and WT plants. Data represent the mean ± SE of three biological replicates. Asterisks (*) indicate statistically significant values greater than the WT at $p \leq 0.05$, using a two-way ANOVA test for variance.

4.3.5 Drought and its effect on antioxidant levels in the plants

The roles of antioxidant enzymes in the defence mechanisms of the wild type control and mutant plants, under water stress were investigated. As an indicator for SOD activity, the percentage inhibition of water soluble tetrozolium salts (WST) were measured (Figure 4.6a). WT SOD levels increased slightly until day 14 pw after which they decreased to less than 50% at day 21 pw of the initial inhibition rate. SOD levels in line M1 were significantly lower than the WT at the initiation of the drought period (day 0 pw) and increased, together with the M2 and M3 lines, until day 14 pw. Mutant lines showed significantly higher levels of SOD on day 21 pw when compared to SOD levels in the WT plants.

During the process of stress development, the levels of catalase stayed more or less constant in the WT until day 14 pw after which it decreased (Figure 4.6b). Mutant lines M2 and M3, over the course of the first 14 days of the drought period, showed a slight increase in levels of catalase and by day 21 pw M2 had significantly higher levels of catalase compared to the levels in the WT plants. However, mutant line M1, under mild stress conditions, started to increase its levels of catalase to a point at day 14 and 21 pw where the levels were significantly higher than levels compared to the WT plants.

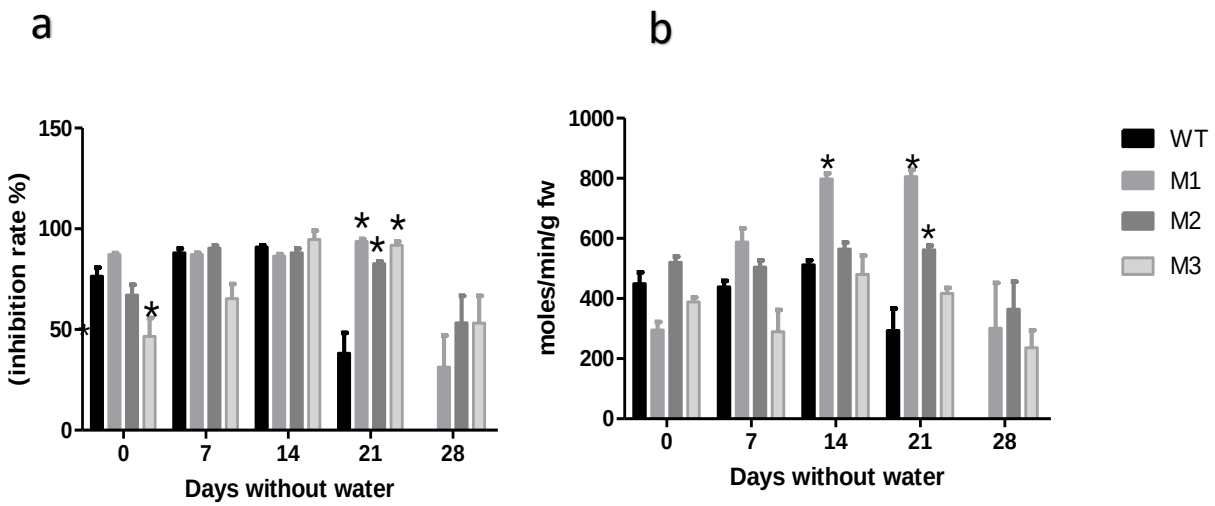

Figure 4.6: Influence of extended drought on **a)** SOD, **b)** catalase enzyme activity in WT and mutant plants. Data represents the mean ±SE of three biological replicates. Significant difference was determined by comparing the WT to the mutant lines at different time points, where values greater than the WT at $p \leq 0.05$, calculated using a 2-way ANOVA, is indicated by (*).

4.3.6 ROS accumulation in plants due to drought

ROS (reactive oxygen species) production in droughted WT and mutant plants were investigated through DAB and NBT histochemical staining of leaf material (Figure 4.7). Under normal physiological conditions WT and mutant lines displayed low levels of H_2O_2 as seen from the light brown staining of the leaf discs on day 0 pw (Figure 4.7a). The levels of ROS then gradually increased as the drought was extended over 21 days, seen in the WT samples with the increase in brown staining on day 14 pw. Two of the mutant lines, M1 and M2, seem to maintain low levels of H_2O_2 even at day 21 and 28 pw, when compared to the WT plants. However, mutant line M3 seem to start accumulating H_2O_2 as early as day 7 pw.

Superoxide also accumulated during the course of the drought period in especially the WT lines, seemingly at higher levels than any of the mutant lines (Figure 4.7b).

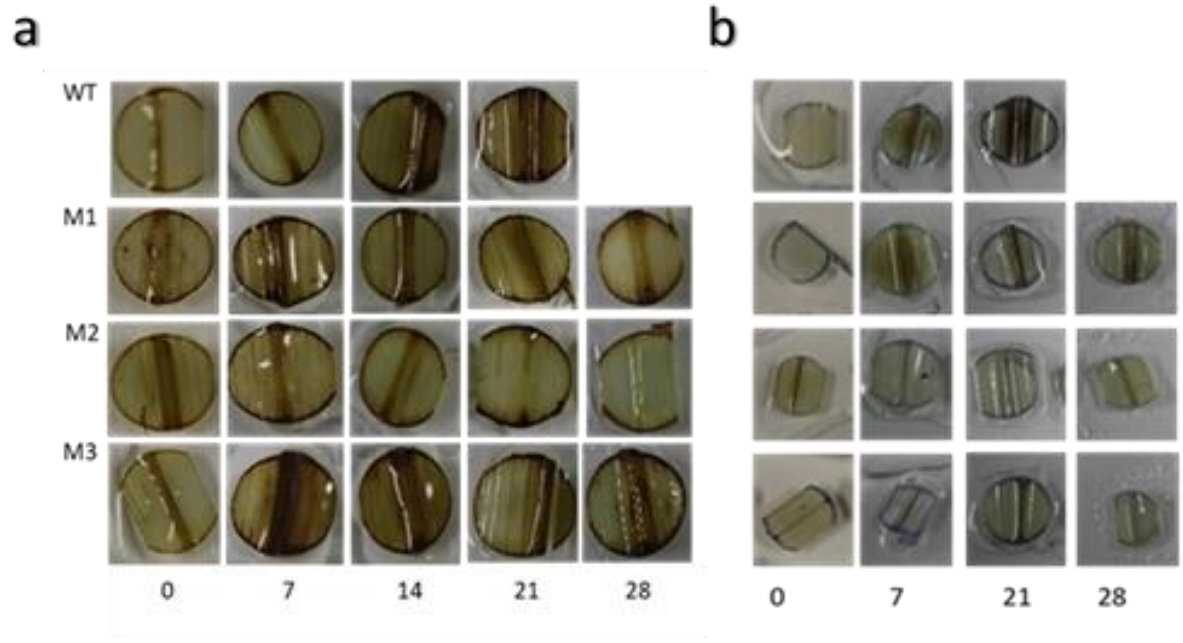

Figure 4.7: Reactive oxygen species accumulation assay. Histochemical staining through **a)** DAB (H_2O_2) and **b)** NBT (O_2^-) staining of leaf material harvested from WT and mutant plants (lines M1, M2 and M3) during a 28 day drought trail. Data is a representation of leaf discs collected from three plants of each line at each time point.

4.3.7 Lipid peroxidation and membrane damage in plants under drought conditions

MDA content in WT and mutant plants, exposed to water stress, was monitored to determine lipid peroxidation levels (Figure 4.8a). Oxidized lipids produce MDA as a decomposition product. In WT

plants, MDA levels seemingly increased from day 0 pw to day 7 pw under mild stress conditions followed by a decrease from day 7 to day 21 pw under severe stress conditions (Figure 4.7a). At the initiation of the drought trial (day 0 pw), mutant lines M1 and M3 showed significantly lower levels of MDA when compared to the WT, and all three mutant lines maintained these significantly lower MDA levels up till day 7 pw. By day 21 pw, all mutant lines showed higher MDA levels when compared to the WT plants, with the levels in lines M2 and M3 being significantly higher than the WT MDA levels.

In terms of electrical conductivity (EC), plants of all lines showed an increase over the drought period, which is to be expected as membranes peroxidation starts to occur due to the drought. However, there were no significant differences in EC levels between the WT and mutant plants across the water stress period. The mutant lines maintained a slightly lower EC under mild stress conditions, up to day 14 pw, but then increased to levels higher than those observed in the WT plants by day 21 pw (Figure 4.8b).

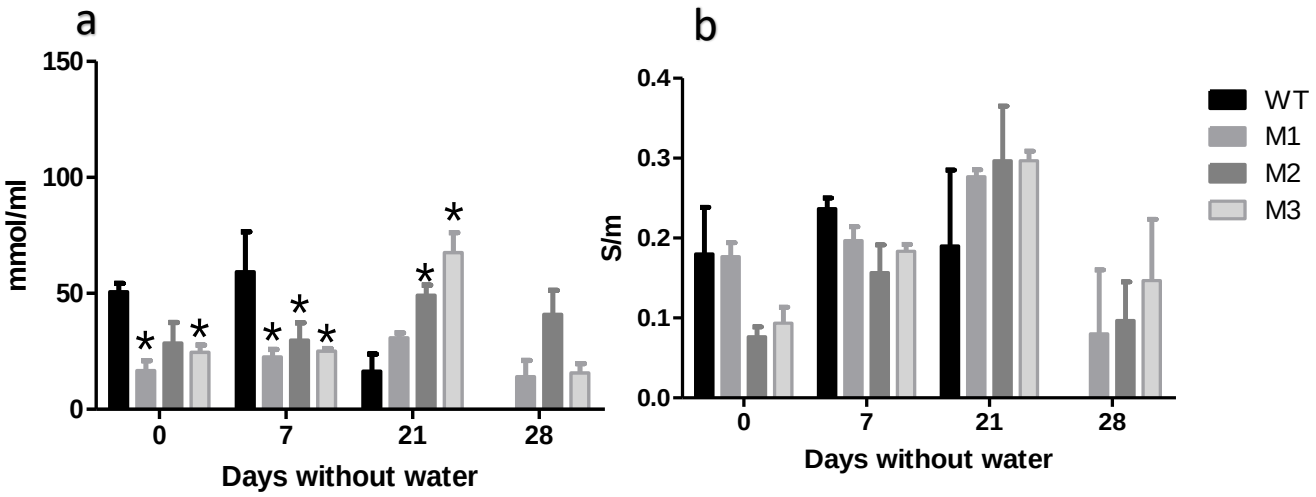

Figure 4.8: Influence of extended drought on **a)** MDA levels, and **b)** electrical conductivity in WT and mutant plants. Data represents the mean ± SE of three biological replicates. Significant difference, where values greater than the WT at $p \leq 0.05$ was determined using a 2-way ANOVA is indicated by (*).

4.3.8 Drought conditions and its effect on proline levels in mutant and WT plants

To establish if the accumulation of osmolytes contribute towards protection against drought in the mutant plants, the production of proline was measured using a ninhydrin-based method (Figure 4.9). The production of proline steadily increased during the first 14 days of stress in all plant lines, both WT and mutant plants (Figure 4.9). The WT plants showed a marked average increase, 4-fold, in

proline levels over this period of mild water stress (first 14 days pw). However, the proline levels across the drought period stayed consistently lower in the all the mutant plants when compared to the WT plants, until day 21 pw. Regardless of the relative high level of variability seen in the proline levels of the WT plants, on day 14 pw both mutant lines M1 and M2 had significantly lower levels of proline accumulated than the WT plants. However, the proline content in the WT plants dropped dramatically by day 21 pw, while the mutant plants maintained slightly higher levels of proline at this stage.

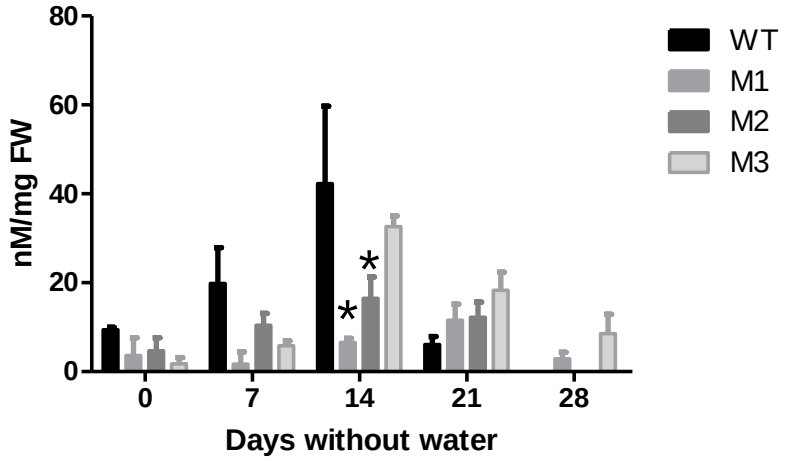

Figure 4.9: Effect of prolonged drought on proline levels in WT and mutant plants. Data represent the mean ± SE of three biological replicates. Asterisks (*) indicate the significance, between the mutant and WT plants, using a 2-way ANOVA test, $p \leq 0.05$.

4.4 Discussion

A number of random mutation induction experiments have been conducted on sugarcane, using either physical (Khan *et al*., 2007; Mirajkar *et al*., 2016; Patade *et al*., 2006), or chemical mutagenesis (González *et al*., 1990; Kenganal *et al*., 2008; Koch *et al*., 2012; Munsamy *et al*., 2013). Mutation induction in these sugarcane lines resulted mostly in changes in salinity tolerance, agronomic traits and disease resistance (Rutherford *et al*., 2014). However, selection of mutated sugarcane lines for drought tolerance through mutation breeding programmes has received limited attention (Khalil *et al*., 2018; Masoabi *et al*., 2017; Rutherford *et al*., 2014). Furthermore, utilizing chemical mutagenesis followed by *in vitro* selection and *in vivo* screening of drought tolerant sugarcane lines has only recently been reported (Khalil *et al*., 2018).

In the past, systems for *in vitro* selection of drought tolerant plant cells or tissues have been established (Rai *et al*., 2011). Still, limited focus has been aimed towards the development of *in vitro*

systems for osmotic tolerance in sugarcane tissue (Errabii *et al.*, 2006; Masoabi *et al*., 2017; Rao and Jabeen, 2013). *In vitro* conditions and all its variables can have complex effects on plant tissue growth and might not lead to optimal or predicted *ex vitro* results. Also, *in vitro* selection of cells for a specific trait might not result in those cells displaying the same response *ex vitro* often due to the reprogramming of gene expression patterns in the different environments (Delporte *et al*., 2012). It is therefore important that mutant plants, identified through *in vitro* selection systems be tested for the trait in greenhouse or field conditions. Therefore, in this current study we characterised putatively drought tolerant mutant lines, generated by Masoabi *et al*. (2017) through EMS mutation induction and *in vitro* osmotic selection, by physiological and biochemical means in glasshouse stress pot trials and determined whether these plants display normal phenotypic characteristic when not under drought conditions.

Firstly, mutant plants were assessed in terms of phenotype and compared to WT plants under non-stress conditions, these differences can potentially affect performance under stress conditions (Blum, 2014). It is known that EMS-induced mutagenesis can influence physiological aspects of mutant sugarcane plants with regards to traits such as tiller and internode numbers, stalk size and leaf number (Gadakh *et al*., 2015; Mahmud *et al.,* 2016). The results presented here indicated that plants from the M3 mutant line were significantly taller with increased leaf length and internode length and showed reduced leaf width and stalk diameter when compared to the WT plants (Table 4.1). Data for the other two mutant lines, M1 and M2, were unfortunately not available due to damage done by interrupted irrigation, these plants were re-planted and are currently growing and will be assessed in the near future. Sugarcane stalk diameter has been seen to influence sugar content (Sinclair *et al*., 2005). In this study the carbohydrate content, referring to sucrose, fructose and glucose levels, in the immature canes of WT plants were almost at the same levels as those in the M3 plants, while sucrose and glucose levels, in the mature cane of the mutant plants were significantly higher than in the WT plants (Figure 4.1).

Secondly, WT and mutant plants were exposed to drought and the phenotypes monitored and relevant physiological and biochemical responses were assessed. Seemingly, mutant plants tolerated water stress better than the WT plants. On average, mutant plants (M1, M2 and M3 lines) survived 5, 10 or 12 days longer without water when compared to the WT plants, which had already died by day 19 pw (Figure 4.2). Over the prolonged drought period of 21 days, dry root mass stayed almost constant in plants from the M1 line, while fold increases of 2.2, 2.8 and 2.2, respectively were seen for WT, M2 and M3 plants (Figure 4.3). In sugarcane, it is known that root development can be influenced by water stress, as seen in this study, but relatively less than the above ground biomass (Smit and Singels, 2006). In the past, an increase or decrease in root dry weight has been associated

with drought tolerant or susceptible sugarcane varieties, respectively (Madhav *et al*., 2017). Root mass can be an indication of root water use efficacy (WUE), which refers to the biomass produced from a unit of water that has transpired (Far *et al*., 2016; Zegada-Lizarazu and Lijima, 2005). In addition, a deep and large root system with a high root density will allow water uptake from deep soil moisture levels (Blum, 2005).

WT plants also showed early signs of senescence with leaf bleaching, rolling and drying occurring from day 14 pw and by day 21 pw plants were completely dehydrated (Figure 4.2). Leaf rolling especially is considered a drought avoidance strategy in sugarcane plants to reduce exposed leaf area to limit water loss through transpiration (Inman-Bamber and Smith, 2005). This was in contrast to the mutant plants which displayed a delay in leaf senescence, for example mutant M2 plants only started to senescence by day 28 pw. Leaf senescence in sugarcane is considered one of the most common responses to water stress (Inman-Bamber and Smith, 2005) and is a regulated breakdown of cellular structure and redistribution of degraded products to other plant organs, such as apical leaves and seeds, which lead to cell death (Buchanan-Wollaston, 1997).

A consequence of leaf senescence is chloroplast degradation (Springer *et al*., 2016). Total chlorophyll content is known to decrease when plants are exposed to drought (Khayatnezhad *et al.*, 2011). In this study, the total chlorophyll content decreases in the WT and M1 plants by day 21 pw on average 1.9 and 1.8-fold, respectively. On the other hand, the chlorophyll content in M2 plants increased 1.2-fold, while M3 plants showed large variation in chlorophyll levels between individual plants (Figure 4.5c). this could be due to the acetone extraction not extracting all present chlorophyll due to experimental error. Only when the plants experienced an extended stress period of 28 days without water did the chlorophyll content drop severely in all plants, but some M3 plants were able to still maintain significantly higher levels when compared to the WT plants. Chlorophyll plays a role in photosynthesis by allowing plants to absorb energy from light (Bennett, 1983). In previous studies, conducted on drought susceptible and tolerant sugarcane varieties, it has been seen that higher chlorophyll leaf content helped sugarcane to tolerate drought better (Ferreira *et al*., 2017).

Photosynthesis is one of the vital physiological processes of plants and is highly sensitive towards available water levels in the soil (Xu and Zhou, 2011). Exposure of plants to water limiting conditions decreases the rate of photosynthesis due to stomatal behaviour changes and chlorophyll degradation due to ROS formation (Aarti *et al*., 2006), which will lead to plant death due to a lack of sugar accumulation and oxygen production (Chaves *et al*., 2008; Inman-Bamber and Smith, 2005). The analysis of chlorophyll fluorescence is considered a sensitive method for the detection and quantification of stress-induced changes in the photosynthetic apparatus (Maxwell and Johnson,

2000; Molinari *et al*., 2007) through the use of maximal photochemical efficiency of PSII (photosystem II) photochemistry (Fv/Fm) (Force *et al*., 2003). Multiple studies have noted reduced Fv/Fm fluorescence when sugarcane plants experienced water stress (Graça *et al*., 2010; Inman-Bamber and Smith, 2005; Ribeiro *et al*., 2013; Silva *et al*., 2007). In this study, the chlorophyll fluorescence ratio (Fv/Fm) in the WT and M1 plants decreased suddenly and severely from day 10 pw to non-functional levels at day 18 and 21 pw, respectively. However, mutant lines M2 and M3 were able to retain constant levels of chlorophyll fluorescence throughout the whole stress period (Figure 4.5b).

Stomatal conductance is an indication of the exit of water and entrance of carbon dioxide in plant cells and as such serves as a signal of the functionality of the stomata (Gimenez *et al*., 2005). Under water limiting conditions the stomatal conductance is influenced by the opening or closing of the stomata through changes in turgor pressure as well as the proper influx and efflux of gases and the correct movement of minerals such as K^+ and Ca^- (Gimenez *et al*., 2005). Stomatal closure can be used as an adaptation to limit the amount of water lost through transpiration due to drought (Pirasteh-Anosheh *et al*., 2016). In sugarcane under mild water stress, stomatal conductance will decrease (Da Graca *et al*., 2010; Ribeiro *et al*., 2013). In Figure 4.5a, it can be seen that in all plants, WT and mutant, the stomatal conductance dropped over the drought period. Only at the later stages of the drought period were the M3 mutant plants able to maintain higher levels of stomatal conductance than the WT plants.

During the drought pot trial, the soil moisture content in all pots decreased on average with a massive 87% by day 21 pw (Figure 4.4). In addition, during the drought period the RWC decreased in all plants. However, the RWC of the WT plants dropped by 40% by day 21 pw, while all the mutant plants were able to maintain higher relative water levels in their leaves, and only lost 40% for M1, and 8% for M2 and M3 by day 28 pw (Figure 4.4). RWC at a level of lower than 30%-40% is detrimental to a plant and can lead to a decrease in the uptake of carbon dioxide and a decrease in respiration (Georgieva and Mihailova, 2016). The RWC has been seen to indicate a plant's ability to survive drought by using water efficiently for metabolic processes and transpiration, thus a higher RWC during drought is an indication of a plant's leaf water status and could represents the capacity for osmotic adjustment (Blum, 2014; Hassanzadeh *et al*., 2009).

Proline is an osmolyte that can play a role in osmotic adjustment or form part of the antioxidant defence system in plant cells (Iskandar *et al*., 2011). It is an amino acid which increases in concentration under abiotic stress, including drought, high salinity, UV radiation and environmental pollutants (Hayat *et al*., 2012). This compound acts as a protector of cells, assists in recovery, stabilises structures such as membranes, is active as scavenging molecule for free radicals, and can act as a buffer with regards to cellular redox potential (Hayat *et al*., 2012). In sugarcane exposed to drought,

proline levels are known to increase to accommodate the higher production of ROS, and correlated with water stress tolerance in various sugarcane cultivars (Ferreira *et al.,* 2017; Suriyan and Chalermpol, 2009; Molinari *et al*., 2007). However, the opposite is also true, where Iskandar *et al.* (2011) found no accumulation of proline in sugarcane under water deficit conditions. In this study, proline levels were consistently higher in the WT plants across the drought period, only at the late stages of the stress, day 21 pw, the proline levels in the WT plants dropped below those observed in the mutant lines (Figure 4.9).

In a further experiment the accumulation of reactive oxygen species (ROS) were evaluated. ROS, also referred to as free radicals, are molecules or atoms that have lost an electron and thus behave as very reactive species (Sharma *et al*., 2012). ROS are also a toxic by-product from oxidative stress that can accumulate due to drought (Gill and Tuteja, 2010). In general, plants maintain low levels of ROS as part of their metabolic processes in non-stress conditions, which can then quickly increase due to abiotic stress (Sharma *et al*., 2012). When present in plants at high levels, free radicals can damage DNA, proteins and lipids, leading to cell death (Lobo *et al*., 2010). In this study the levels of H_2O_2 and O_2^- were determined visually using two staining procedures which utilised DAB and NBT. These staining assays do not quantify the amounts of free radicals but serve only as an indicator of growing amounts of hydrogen peroxide and superoxide under stress conditions (Liu *et al*., 2014; Grellet Bournonville and Díaz-Ricci, 2011). H_2O_2 and O_2^- levels seemed to increase in the WT and M3 plants, while lower levels were seen in the M1 and M2 plants (Figure 4.7), which might indicate differences in the plant line's ability to combat oxidative stress by ROS scavenging. In the past, increases in H_2O_2 levels in young sugarcane plantlet have been observed in relation to water stress (Boaretto *et al*., 2014). The blue precipitate around the edges of the leaf discs might be due to increasing ROS levels as a result of wounding and not water deficit stress when cutting the discs. In the future this experiment should be repeated with intact leaf material.

Enzymatic antioxidants such as SOD are the first defence line against ROS accumulation in plants under stress (Ighodaro and Akinloye, 2017; Alscher, 2002). SOD acts as a ROS scavenger and converts O_2^- with the help of metal cofactors into O_2 and H_2O_2, a less reactive peroxide (Ighodaro and Akinloye, 2017). H_2O_2 in turn is then detoxified by enzymes such as CAT (Wu and Cederbaum, 2003). In this study, SOD levels increased slightly in all plants under mild water stress, with the exception of mutant line M1 which remained at constant levels during this period (Figure 4.6a). However, by day 21 pw the SOD levels in WT plants decreased 2-fold, while all the mutant lines were able to maintain significantly higher levels of SOD at this late stage of the stress period. By maintaining high levels of SOD for longer, damage to cellular structures can be minimised (Alscher, 2002). SOD in sugarcane under drought is

genotype-dependant, but has been shown to increase, as with other antioxidants, in an attempt to combat ROS accumulation (Ferreira *et al*., 2017; Madhav *et al*., 2017).

Catalase levels in the M1 mutant line increased 2.7-fold by day 14 pw, and remained at these significantly higher levels till day 21 pw when compared to the WT plants (Figure 4.6b). Mutant lines M2 and M3 showed a slight increase in CAT level between day 0 and day 14 pw and maintained these higher levels, when compared with the WT plants, at the later stages (day 21 pw) of the drought period. Catalase is also an antioxidant that is known to increase in plants due to oxidative stress, caused by stresses such as drought, and has the ability to combat ROS formation (Mhamdi *et al*., 2010; Zhang and Kirkham, 1994). In sugarcane, catalase has been seen, under normal conditions, in cells in the cytoplasm and peroxisomes as well as to a lesser extent in mitochondria where it functions by scavenging H_2O_2 molecules in an effort to limit the damaged caused by this molecule (Sun *et al*., 2018). However, according to literature, it is still not clear what the connection is between drought and catalase production in sugarcane (Ferreira *et al*., 2017).

To further investigate oxidative stress due to water deficit in the WT and mutant lines, the malondialdehyde (MDA) content was measured. MDA is a product of lipid membrane peroxidation and is an indicator of oxidative damage (Ayala *et al*., 2014; Kong *et al*., 2016; Weitner *et al*., 2016). Lipid peroxidation is caused by ROS accumulation which results in changes in membrane structure and membrane damage (Hodges *et al*., 1999). Low levels of MDA have been associated with drought tolerance in plants such as wheat and rice as well as sugarcane (Abbas *et al*., 2014; Sultan *et al*., 2012; Zu *et al*., 2017). Results from the current study indicated that the MDA content in all the mutant lines remain at significantly lower levels during mild drought, up to day 14 pw, when compared to the WT plants indicating less oxidative damage during mild stress conditions (Figure 4.8a). However, MDA levels in the mutant lines increase to significantly higher levels than in the WT plants at the severe, later stages of the drought period, even though the WT showed higher levels of leaf senescence (day 21 pw). This shows that the mutant lines maintained their membrane integrity for a longer period of time. Electrical conductivity, as a further indicator of membrane damage, showed an increase in all mutant plants up till day 21 pw. During the same period, EC levels in the WT plants remained relatively constant at lower levels than seen in the mutant plants (Figure 4.8b). Membrane damage is detrimental to the plant due to electrolytes leaking out of cells and into the surrounding spaces (Li *et al*., 2013). Thus, an increase in membrane damage can lead to the death of plants due to a lack of components in cells that are necessary for normal growth and development and cellular reactions (Van Breusegem, 2006).

In conclusion, under non-stress conditions M3 mutant plants showed no detrimental growth defects, and even produced more sucrose in mature stalk material. Under water deficit conditions, all mutant plants were able to survive longer than the WT N19 plants and maintained higher relative water content levels in their leaf tissue. This study then analysed physiological and biochemical modifications in these mutant plants that are considered suitable traits to distinguish between drought susceptible and tolerant sugarcane genotypes, such as assessment of the photosynthetic apparatus, osmolyte, antioxidant and ROS accumulation, and lipid peroxidation. There were marked differences when the WT plants were compared to the plants from the different mutant lines under drought conditions even though substantial variation between plants within a specific line were sometimes observed. In the majority of mutant lines, the photosynthetic machinery was active for longer periods during water stress, which included the maintenance of stomatal conductance and the chlorophyll fluorescence ratio. There seemed to be less ROS accumulating in some of the mutant lines, which might be a result of the significantly higher levels of enzymatic antioxidants, specifically SOD and CAT, seen accumulating under severe water stress conditions, as experience by the mutant plants at 21 days without water. Lower levels of ROS might also explain the lower observed levels of MDA in the mutant plants under mild water stress conditions. Overall, the mutant sugarcane plants generated through chemical mutagenesis and identified through an *in vitro* osmotic selection system by Masoabi *et al*. (2017) display enhanced drought tolerant phenotypes.

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Conclusions

Currently, South Africa is experiencing the effect of global warming, with increases in temperature linked to the devastating increase in the severity and frequencies of droughts in the area. Overall, water as a resource, including its use for agriculture, is becoming scarcer and crop plants experience water stress due to decrease precipitation and less water being available for irrigation. Thus, the country needs to focus on ways to increase the tolerance of our crop plants in terms of drought.

Sugarcane is an important crop plant in South Africa, harvested mainly for its sugar content, for both the local and export markets, as well as its bagasse component, which contributes to alternatives to fossil fuel sources. In addition, the sugarcane industry creates jobs and contributes financially to the lives of the families working in this particular industry pipeline. Currently, in South Africa, only 15% of arable land for sugarcane production is irrigated, while the remaining 85% is rain-fed. The rain-fed KwaZulu Natal region, accounts for 80% of the country's sugarcane production but in years of water scarcity, yields are severely compromised. Thus, cultivating new sugarcane cultivars which are able to withstand the changes in rainfall and precipitation and can be seen as drought tolerant is of the utmost importance. However, traditional breeding methods in sugarcane are considered challenging due to the cane's ploidy nature, its genome that has not yet been fully annotated and limitations in seed fertility. Therefore, additional methods have to be investigated in an effort to develop useful traits in sugarcane, especially enhancing drought tolerance.

Mutation breeding originated in the 1900's and can be used to increase genetic diversity in crop plants. Through mutagenesis, random mutations are induced in the genome of a plant without having any prior knowledge to the placement of the mutations, resulting in desirable traits without compromising the plants' growth and development. Physical mutagenesis can be induced through ionizing or non-ionising radiations, of which the most popular methods include gamma rays, X-rays and neutron ion beams. Physical mutagenesis is known to mostly induce directly double-stranded breaks, point mutations and deletions in the DNA structure of the exposed organism, but also secondary damage due to the formation of free radicals as a result of ionisation of water molecules present in the irradiated tissue. Gamma radiation has been used in the past to induce mainly salt tolerance and sucrose accumulation traits in sugarcane mutation breeding attempts.

A physical mutagenesis experiment was performed where sugarcane callus from the NCo310 and N58 cultivars were generated and exposed to gamma radiation at dosages that vary between 10 and 40 Gy. An ideal dosage should be sufficient to ensure high mutation frequency but induced mutations should not bypass the threshold that would result in plant cell death. In order to identify potential useful mutations, irradiated sugarcane callus was placed on *in vitro* growth media containing

PEG6000, which selects for cells with enhanced osmotic stress tolerance. Callus growth, embryogenicity and cell death of the calli irradiated at different gamma dosages, were monitored. Overall, only one *in vitro* plantlet, from N58 callus originally irradiated at 20 Gy, was able to survive the selection and mature to form roots. In the future larger amounts of explant tissue will need to be radiated to ensure the induction/selection of potential drought tolerant mutants, since the efficiency of gamma radiation to induce useful mutations linked to a specific phenotype seems to be limited even across multiple radiation dosages.

In a histological experiment, irradiated callus and leaf disc tissue were also assessed for the formation of micronuclei, which can act as a biomarker of genotoxicity, indicative of induced DNA damage at the different radiation dosages. Micronuclei are chromosome fragments, identified by their nucleus-like structure, that are left behind during cell division which is then covered in a nuclear membrane and are not included in the main nucleus. Reports in literature link the formation of micronuclei to radiation dosage increases. Thus, micronuclei can be an indication of the amount of damage cells have undergone while being irradiated. In this study we first had to develop a histological protocol to view micronuclei in sugarcane before the quantitative assessment of these structure link to potential radiation damage. Micronuclei have never been detected in sugarcane before, and this was therefore seen as a novel and new approach to assess radiation damage. It was determined that indeed radiation induced the formation of micronuclei, while control samples showed no micronuclei formation. However, no linear correlation could be made between irradiation dosages and the number of micronuclei formed.

Through chemical mutagenesis, by exposing calli from the N19 cultivar to the chemical EMS and *in vitro* selection for osmotic stress tolerance, Masoabi *et al*. (2017) generated putatively drought tolerant mutant sugarcane lines. EMS has been used to induce beneficial mutations in other crop species, but rarely in sugarcane and not for an increased drought tolerant phenotype. In a final experiment, the level of drought tolerance in these mutant lines were assessed through drought glasshouse pot trials.

A mutant line (M3) displayed at maturity, under normal growth circumstances, no detrimental growth defects and even produced more sucrose in mature stalk material. The plants of two of the assessed mutant lines were lost due to interrupted irrigation and the assessment of their growth under non-stress conditions will need to be repeated in the future. Under water deficit conditions, all mutant plants, lines M1, M2 and M3, were able to survive longer than the wild-type control N19 plants and maintained higher relative water content in their leaf tissue. Furthermore, physiological and biochemical modifications in these mutant plants that are considered suitable traits linked to drought

tolerant sugarcane genotypes, such as assessment of the photosynthetic apparatus, osmolyte, antioxidant and ROS accumulation, and lipid peroxidation, were assessed. There were marked differences when the WT plants were compared to the plants from the different mutant lines even though substantial variation between plants within a specific mutant line were sometimes observed. In the majority of mutant lines, the photosynthetic machinery was active for longer periods under water deficit condition, which included the maintenance of stomatal conductance and the chlorophyll fluorescence ratio. There seemed to be less ROS accumulating in some of the mutant lines, which might be a result of the significantly higher levels of enzymatic antioxidants seen accumulating under severe water stress conditions. Lower levels of ROS might also explain the lower observed levels of MDA in the mutant plants under mild water stress conditions. Overall, the mutant sugarcane plants generated through chemical mutagenesis and identified through an *in vitro* osmotic selection system display enhanced drought tolerant phenotypes. In the future, the enhanced drought phenotype in these mutant lines should be assessed under field conditions where the plants will be exposed to conditions subjected to natural fluctuating precipitation, which might include irregular, extended or overall limited water throughout the plants' growth cycle.

Supplementary files

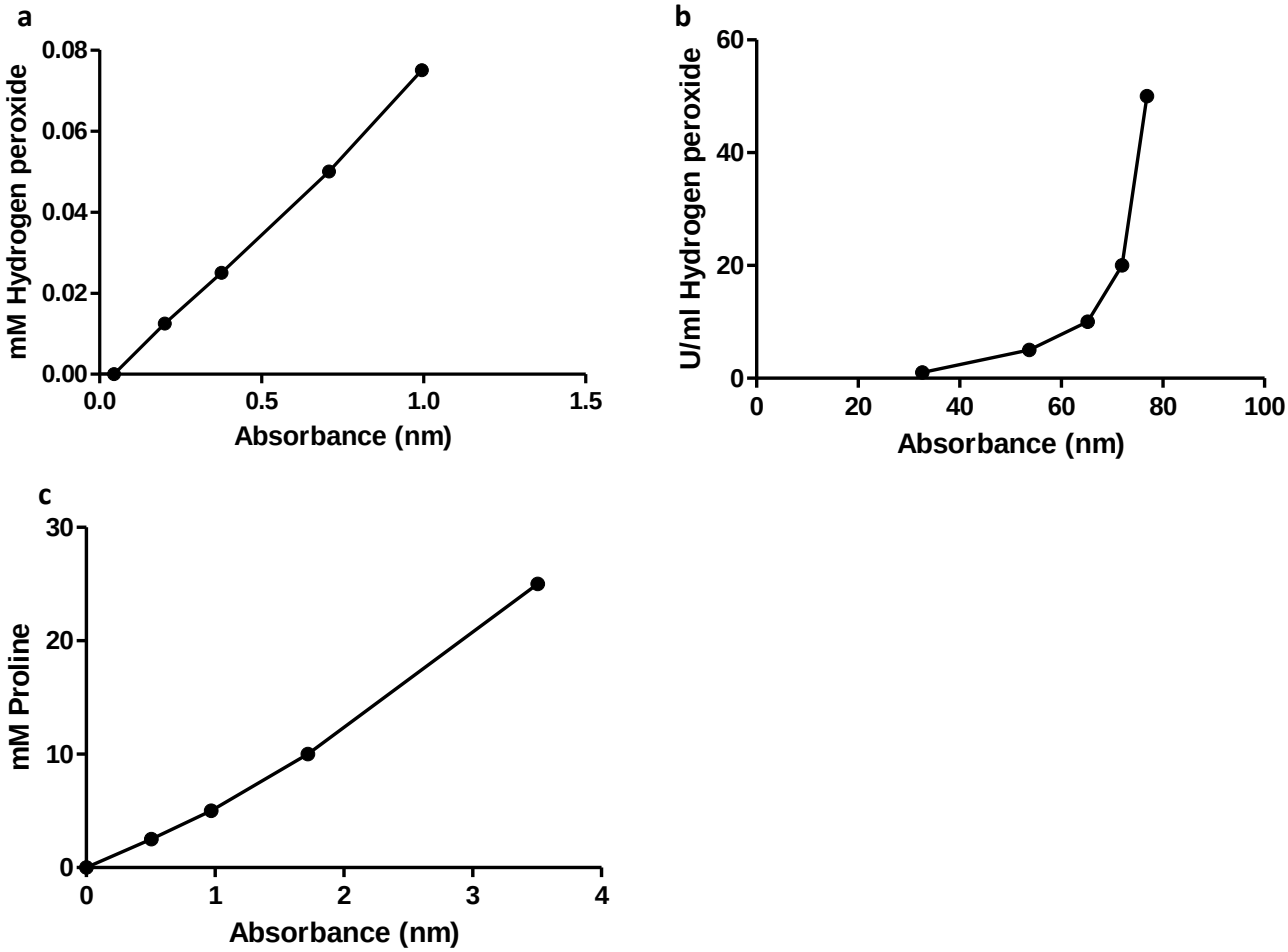

Figure 6.1: Graphs used to determine the standard curves for **a)** Catalase according to the absorbance of red quinoneimine dye at OD_{520} as described in the Catalase activity kit (Sigma-Aldrich), where H_2O_2 concentrations ranged from 0-7.5 mM; **b)** SOD according to the absorbance of formazan dye at OD_{440} as described in the SOD kit (Sigma-Aldrich), where SOD concentrations ranged from 0.05-200 mM; **c)** Proline according to the absorbance of L-Proline at OD_{520} as described by Bates *et al*. (1973) where L-Proline concentrations ranged between 5-100 mM.

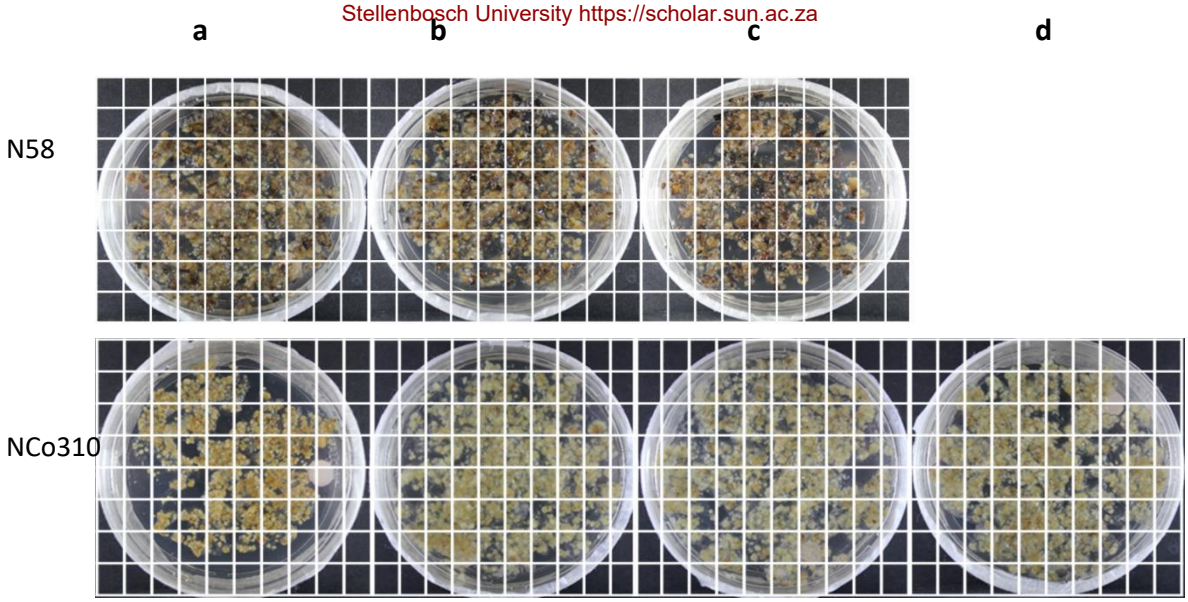

Figure 6.2: N58 sugarcane callus after 8 weeks in the dark, on semi-solid MS3 containing PEG6000, after irradiation with different gamma ray dosages. N58: **a)** Control **b)** 20 Gy **c)** 40 Gy; NCo310: **a)** Control **b)** 10 Gy **c)** 20 Gy **d)** 40 Gy. Gridlines were used to determine the totale coverage in cm^2.

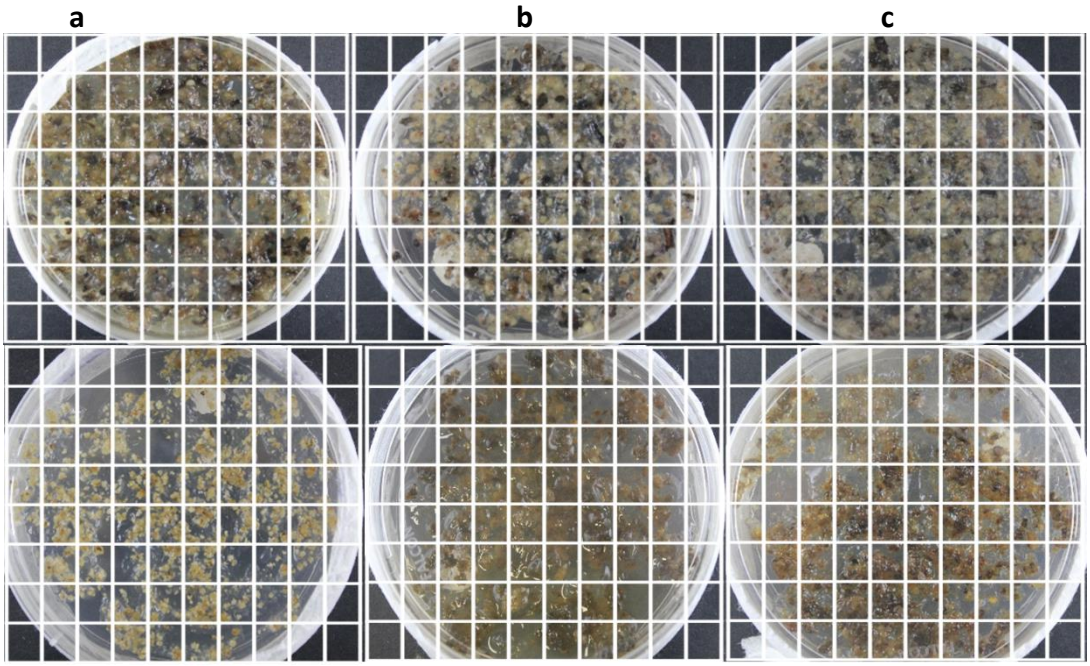

Figure 6.3: N58 callus samples cultured for 6 weeks in a light growth room on semi-solid MS medium containing 20% PEG6000 after exposure to different gamma ray dosages **a)** Control **b)** 20 Gy **c)**40 Gy. Gridlines were used to determine the totale coverage in cm^2.

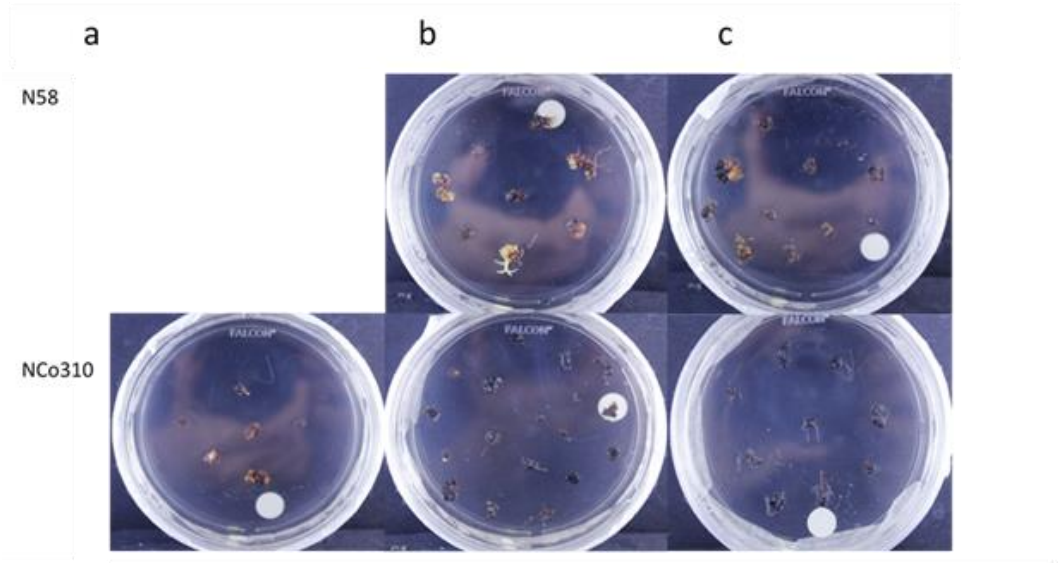

Figure 6.4: NCo310 plantlets formed after 10 weeks in the light on semi-solid MS media, after 20% PEG6000 selection following gamma radiation at dosages: **a)** 10 Gy **b)** 20 Gy **c)** 40 Gy.

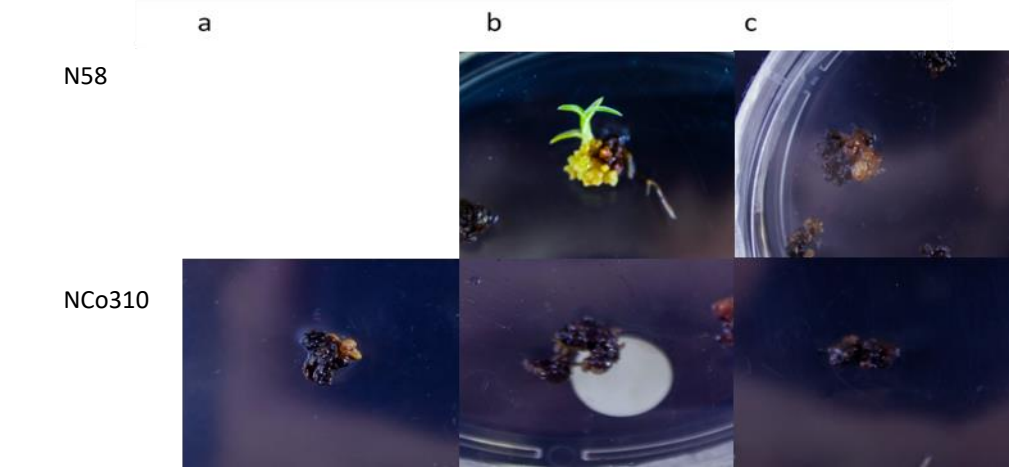

Figure 5.5: Close-up of N58 and NCo310 plantlets formed after 10 weeks in the light on semi-solid MS media, after 20% PEG6000 selection following gamma radiation at dosages: N58: NCo310: **a)** 10 Gy **b)** 20 Gy **c)** 40 Gy.